



# Fullerene: biomedical engineers get to revisit an old friend

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**In 1985, the serendipitous discovery of fullerene triggered the research of carbon structures into the world of symmetric nanomaterials. Consequently, Robert F. Curl, Harold W. Kroto and Richard E. Smalley were awarded the Noble prize in chemistry for their discovery of the buckminsterfullerene (C<sub>60</sub> with a cage-like fused-ring structure). Fullerene, as the first symmetric nanostructure in carbon nanomaterials family, opened up new perspectives in nanomaterials field leading to discovery and research on other symmetric carbon nanomaterials like carbon nanotubes and two-dimensional graphene which put fullerenes in the shade, while fullerene as the most symmetrical molecule in the world with incredible properties deserves more attention in nanomaterials studies. Buckyball with its unique structure consisting of sp<sup>2</sup> carbons which form a high symmetric cage with different sizes (C<sub>60</sub>, C<sub>70</sub> and so on); however, the most abundant among them is C<sub>60</sub> which possesses 60 carbon atoms. The combination of unique properties of this molecule extends its applications in divergent areas of science, especially those related to biomedical engineering. This review aims to be a comprehensive review with a broad interest to the biomedical engineering community, being a substantial overview of the most recent advances on fullerenes in biomedical applications that have not been exhaustively and critically reviewed in the past few years.**

## Introduction

Carbon is one of the most abundant elements in nature and definitely holds one of the first places in nanomaterials research field. Entering the carbon-based nanomaterials world was triggered by the discovery of buckminsterfullerene (shortened to fullerene or buckyball), the third carbon allotrope, after graphite

and diamond, in 1985 by Robert F. Curl, Harold W. Kroto and Richard E. Smalley [1]. Since geodesic domes designed by Buckminster Fuller in 1960s provided clues to realize fullerene C<sub>60</sub>'s structure, this molecule was named after this American architect, buckminsterfullerene. After this discovery, in 1990, Wolfgang Krätschmer of the Max Planck Institute for Nuclear Physics, and Donald Huffman of the University of Arizona, with their students, prospered in producing fullerene in bulky quantities

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[2]. Finally, in 1996 Kroto, Smalley and Curl were awarded the Noble Prize in Chemistry for fullerene discovery.

Nowadays, we know fullerenes exist in nature and interstellar space [3,4]. In the past, fullerene was Molecule of the Year in 1991, and has attracted majority of research projects than other scientific subjects, to the extent that average rate of publications was every thirteen hours in first few years after its discovery [5].

Nanodiamond which is a significant member in carbon nano-materials family has been discovered before fullerene in 1963–1982. Indeed, fullerene could be considered as the first discovery among symmetric carbon nanostructures [6–8], and its discovery revealed new perspectives in carbon-based materials that resulted in research on carbon nanotubes, cylindrical cousin of buckyball, and rapid developments in advanced materials field.

Although the emergence of 2D carbon nanomaterials, as graphene reported by Geim and Novoselov in 2004, who were awarded the Noble Prize in 2010, marginalized fullerenes and moved the majority of the studies towards other carbon nanostructures [9,10], the incredible properties of fullerene could not be underestimated.

Fullerene, with its unique structure consisting of  $sp^2$  carbons, presents a high symmetric cage with different sizes ( $C_{60}$ ,  $C_{76}$  and so on). The most abundant fullerene in the as-synthesized composition is  $C_{60}$ , the molecular structure of which, together with the one of  $C_{70}$ , can be observed in Fig. 1 [11].  $C_{60}$  consists of 60 carbon atoms with  $C_5-C_5$  single bonds which form 12 pentagons and  $C_5-C_6$  double bonds which form 20 hexagons [1]. Indeed, each fullerene with  $2n + 20$  carbon atoms contains ' $n$ ' hexagon [12]. As shape, fullerene  $C_{60}$  is like a soccer balls, and Yadaf *et al.* [13] have stated that the ratio of fullerene molecule to soccer ball is the same as the ratio of soccer ball to the earth, where the diameter of the earth, soccer ball and fullerene molecule are  $12.75 \times 10^6$  m,  $2.2 \times 10^{-1}$  m and  $7.0 \times 10^{-10}$  m, respectively.

In relation to crystallographic properties of fullerene molecule, the presence of symmetric elements including 30 twofold axes, 20 threefold axes and 12 fivefold axes has made fullerene the most symmetrical molecule (as already mentioned) that is controlled by Golden Mean rule [13,14].  $C_{60}$ , with face centered cubic lattices (FCC) in solid phase, has a stable structure to the extent that the destruction of fullerene cage happens at temperatures higher than  $1000^\circ\text{C}$ . Divergent spectroscopic methods like Raman spectroscopy, UV-vis, NMR and FTIR could be deployed to characterize

fullerene [11]. Moreover fullerene results to be a compatible nano-material with biomolecules specially which are structured by Fibonacci sequence and possess Golden Mean properties too [15]. Among the different characteristics of this molecule,  $C_{60}$  has the ability to produce oxygen species in exposure of visible light and makes it a suitable candidate for photodynamic therapy [16]. The interesting behavior of fullerene in solutions presents a unique solvent-solute interaction in which rigid molecular structure of fullerene has not been undergone conformational and solvent-dependent changes [17]. Solubility of fullerenes has been investigated with discrepant experiments, nevertheless, fullerenes' solubility is not predictable and there is no reliable theory to explain fullerenes' behavior in various solvents [18].

Pristine  $C_{60}$  has very low solubility in water; however, it can form aggregates in water solutions and make stable colloid solutions which contain both individual fullerene and fullerene clusters [19]. Ruoff *et al.* showed that fullerene solubility is a function of several factors such as refractive indices, dielectric constants and molecular volumes. The dielectric constant of water at room temperature is approximately 80, which is high, index of fraction of water at room temperature is 1.33 and its molar volume is 18 ml/mol and these index fraction and molar volume of water are small for fullerene solubility [17,20].

The hydrophobic nature of fullerene makes its solubility in organic (especially aromatic) solvents like toluene, chloroform and benzene quite high [21,22], while in polar solvents it is very challenging, and this is critical for biological applications. A number of methods have been developed in order to boost fullerene solubility in water like preparing two-phase colloidal solutions, synthesizing fullerene derivatives and fullerene polymers [23,24]. Although hydrophobicity of fullerene provides it with some degrees of compatibility in biological systems, hydrophilicity of materials in biological environments has greater importance in comparison with hydrophobicity.

As mentioned above, different strategies have been exploited in order to promote fullerenes' behavior in solvents, especially aqueous dispersions, which play a crucial role in biological applications [25–27]. One of them is involved with transferring fullerenes from an organic solution such as toluene or benzene to an aqueous phase with ultrasound treatment with following removal of the organic solvent [21,28]. The mechanism of fullerene dispersal in aqueous solutions with the aforementioned method could be explained in two ways: (i) the formation of water shell via H-bonding and charge transfer between  $C_{60}$  and water molecules stabilizes fullerene in water [19,29] and (ii) ultrasound treatment creates a covalent bond between hydroxyls and carbons in fullerene cage, which culminates in fullerenol moieties and consequent easy fullerene dissolution [19,30]. Other attempts include encapsulation in special carriers like cyclodextrins, calixarenes, polyvinylpyrrolidone, micelles and liposomes, which give advantages for biopharmaceutical applications like drug delivery [31–33]. In addition to the mentioned methods, chemical modification of fullerene with hydrophilic substance such as amino acids, carboxylic acids, polyhydroxyl groups, or amphiphilic polymers enhances hydrophilicity of fullerenes in biological systems [34–36]. Once rendered soluble in biological media, the excellent properties of buckminsterfullerene make it useful for biomedical applications including antiviral activity, antioxidant activity as a

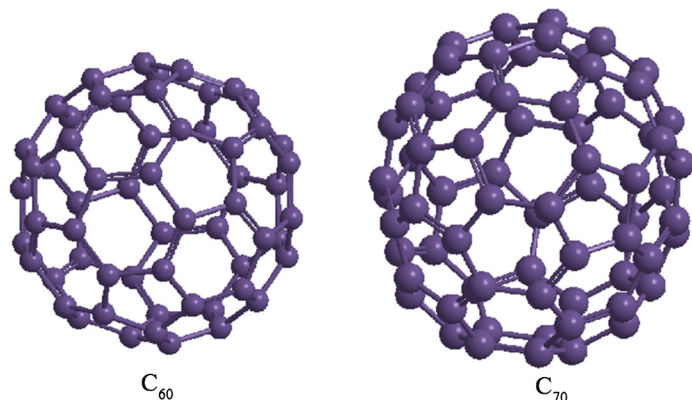


FIGURE 1

Schematic of fullerene molecules ( $C_{60}$  and  $C_{70}$ ).

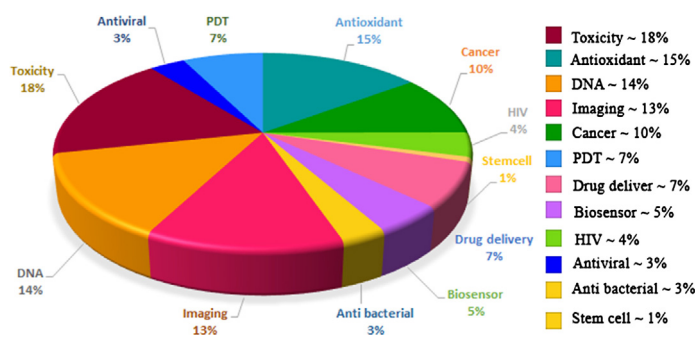


FIGURE 2

Distribution of various biomedical applications of fullerene.

radical scavenger, encapsulating medically materials like magnetic metals for theranostic applications and as a drug carrier in scaffolds [37–39].

In order to investigate divergence of publications regarding the biomedical applications of fullerene, we searched the publications within the scientific community using Scopus database (date of search: 21 November 2016). We used keywords including cancer, photodynamic therapy, antiviral, anti-bacterial, HIV, DNA, RNA, toxicity, biosensor, antioxidant, imaging, drug deliver, stem cell. The papers that have these keywords in their title, abstract and keywords list have been counted and the results of the distribution of them in various biomedical applications are demonstrated in Fig. 2. The number of papers with word ‘toxicity’ is more than other keywords and it demonstrates the great importance that was placed to the toxicity of the fullerenes which is a prerequisite for the biomedical applications of fullerenes.

Now, in the 30th anniversary of  $C_{60}$  discovery, we consider that is time to review the progress of the fullerene-based research from its birth until now. In this article, we will review 30 years of investigations on fullerenes, started in 1985 with a serendipitous discovery. Although the advent of other carbon nanomaterials like the carbon nanotubes and the two dimensional graphene put fullerenes in the shade, this form of carbon, that can be considered a 0D carbon nanostructure, deserves great attention in nanomaterials studies, especially considering that, contrary to the other cited materials, its structure is perfectly define, the reproducibility of its functionalization is not an issue and its characterization can take advantage of both classical and innovative techniques.

## Synthesis of $C_{60}$ fullerene

### Huffman–Krättschmer method

Three main methodologies for production of fullerenes have been utilized, evaporation of carbon, incomplete combustion of benzene in oxygen [40] and microwave method [41]. The first bulky production of fullerene, which has been done by Krättschmer *et al.* [2] in 1990, was based upon evaporation and recondensation of graphite, its schematic is shown in Fig. 3a. Since a requisite for fullerene formation is carbon atoms in gaseous phase [2,5], in this process pure graphite was used as a source of carbon atoms, by heating graphite in helium atmosphere with 100–200 Torr pressure, a light and condensate soot was produced which contained fullerene. In order to extract fullerene, the soot was dispersed in benzene in a Soxhlet extractor and the benzenic solution of fullerene was percolated from the Soxhlet cartridge in the flask

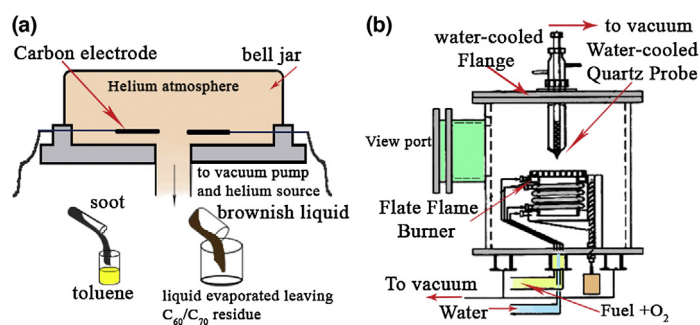


FIGURE 3

Schematic of fullerene synthesis methods: (a) Huffman–Krättschmer method based on evaporation of graphite and (b) premixed chamber in combustion method.

and so separated by the other components [42]. An alternative method for the fullerene extraction included the soot heating in an inert gas or under vacuum followed by sublimation and formation of fullerene [2,40].

In order to vaporize graphite or other sources of carbon like coal, techniques such as arc-evaporation [2], pyrolysis [43], radio-frequency-plasma [44] and laser ablation [45] could be exploited. Although these methods provide higher quantity of fullerene, the arc process results to be the best-established technique for production of carbon soot, and it is more efficient and appropriate for commercial uses. Regardless of the method used to produce carbon soot, fullerene must be purified, most of the times by extraction with organic solvents and, because of limited solubility of fullerene in these solvents, large volumes of solvents are needed to produce acceptable yield of fullerene [5].

### Combustion method

Another technology for producing fullerenes is based upon incomplete combustion of hydrocarbons in sooting flames [46,47]. In 1987, fullerene ions in flames were found and in 1991 Howard *et al.* [48] succeeded in extracting significant yields of fullerenes from laminar sooting flame of premixed mixture of benzene and oxygen at low pressure, the schematic of the premixed chamber is shown in Fig. 3b [48,49]. In this method, flame conditions like pressure, C/O ratio, residence time and gas velocity in combustion of benzene influence fullerene amount and composition [31–34]. Among them, pressure is an influential factor and formation of fullerene could be done at low pressure like the astrophysical atmosphere at temperatures lower than 1700 K [50]. Furthermore, combustions methods including laser pyrolysis of vapor hydrocarbons [46] and laser irradiation of polycyclic aromatic hydrocarbons (PAHs) [51] have been used to produce fullerene.

### Microwave method

In addition to the well-known methods mentioned above, there are technologies exploiting microwave in order to synthesize fullerene. In 1995, Ikeda *et al.* [41] obtained fullerene by using naphthalene and microwave-induced  $N_2$  plasma at atmospheric pressure in a cylindrical coaxial cavity and they reported this method as an effective route to fullerene synthesis. Microwave-induced nitrogen plasma is a noticeable source for excitation and ionization of molecular species like benzene or naphthalene and,

moreover, plasma condition could be easily controlled by adjustment of microwave power [41].

Another use of microwave for production of fullerene has been investigated recently to transform graphite powder to fullerenes. The microwave method possesses advantages over conventional heating methods, providing a homogenous heating of the precursors. In this study it was found that time and temperature did not affect the production of fullerene, while the amount of graphite powder and microwave intensity led to higher yield of synthesized fullerene [52].

### Fullerene functionalization

The unique physical and chemical features of fullerene make it a favorable option for biological and material chemistry applications [39,53,54], however its functionalization is often needed for such purposes. As previously mentioned, despite fascinating properties of fullerene  $C_{60}$ , one obstacle in exploiting this molecule in biological applications is its insolubility in water and low solubility in many organic solvents [26]. The presence of double bonds is the most effective feature exploited to functionalize fullerene. In fact, they can be involved in addition reactions, therefore, the outer sphere of the carbon cage can be modified, and derivatives containing various functional groups can be synthesized [55]. Also, pristine fullerene contains no hydrogen atom and cannot involve substitution reactions, however fullerenes are oxidizing agent, in particular when exposed to UV-visible irradiation can produce active oxygen forms [56].

Two approaches have been used to modify fullerenes: (a) use of solubilizing agent to partially mask fullerene surface, and (b) chemical modification of fullerene via covalent functionalization [39,57,58]. The first can take advantage of linking polymer chains to fullerene [59], entrapping this molecule in cyclodextrins or calixarenes [26,60,61], incorporating into artificial lipid membranes [62,63], inducing co-solvation with polyvinylpyrrolidone in organic solvents [60]. Numerous functionalization routes have been used to enhance fullerene hydrophilicity and also extent its biological and pharmaceutical applications [39]. Alteration of carbon atoms' hybridization from  $sp^2$  to  $sp^3$  is the driving force for addition to fullerene [61]. Various free-radical reactions, cyclopropanation, or cycloaddition reactions such as [1 + 2], [2 + 2], [3 + 2] and [4 + 2] reactions can covalently conjugate various molecules to  $C_{60}$  (for an example see Fig. 4) [36]. Due to the electron deficient nature, fullerene behaves as a  $2\pi$  electron-deficient dienophiles and 1,3-dipolarophiles, which experience a variety of cycloaddition reactions such as [1 + 2], [4 + 2] (Diels-Alder reactions), [3 + 2], and [2 + 2]. A large number of fullerene derivatives have been prepared using different types of cycloaddition reactions [64].

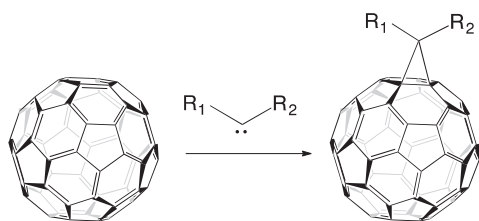


FIGURE 4

Example of a [2 + 1] cycloaddition reaction.

The latter approach, that is, the chemical modification, is an advanced and effective method which resulted in a variety of fullerene derivatives with attached amine ( $-NH_2$ ), hydroxyl ( $-OH$ ) and/or carboxyl groups ( $-COOH$ ) and examples of some of the so-obtained derivatives are shown in Fig. 5 [26,39].

Among the various fullerene functionalizing routes, Hirsch *et al.* [65] succeeded in obtaining great results by attaching carboxylic groups in dendrimeric mode to the fullerene and promoted its solubility in water. Filippone *et al.* [66] also used a covalent functionalization to link  $C_{60}$  and cyclodextrins, with the synthesis of (permethylated- $\beta$ -cyclodextrin)-fullerene conjugate in order to enhance fullerene solubility in water and polar media. Also fullerlenols, which contains hydroxyl group with  $C_{60}(OH)_n$  formula, present a very good water solubility and have been utilized to absorb oxygen free radicals and to reduce neuronal tissue damage [66]. In addition carboxyfullerenes have been exploited in the treatment of neurodegeneration caused by amyotrophic lateral sclerosis (ALS) [67,68].

### Endohedral fullerenes

The fullerene hollow cage allows to use this carbon nanostructure as container for various atoms, ions and molecules and the term 'endohedral' is used to indicate such fullerene species. Endohedral metallofullerene is a captivating class of fullerenes which modifies electric and magnetic properties of fullerene cage and extends applications of this carbon allotrope in different fields especially in the realm of biomedicine.

### Synthesis of endohedral fullerene

Until now, various methods have been developed for the production of endohedral fullerenes in macroscopic scale, similarly to the fullerene preparation strategies, with the creation of carbon-rich plasma in atmosphere containing inert gas like He or Ar. These techniques include vaporization of graphite with arc discharge [69–71], laser ablation [72–76], radiofrequency furnace [77] and resistive heating [78], implantation of the atoms through the carbon cages' walls via ion bombardment [79–81], high pressure treatment [82–84] and the last technique is the chemical routes by opening orifices in the fullerenes [85,86]. Among these methods, arc discharge is the most favorable for production of endohedral fullerene [87].

Initially, endohedral metallofullerenes (EMFs) were produced by laser ablation. In this process, a composite disk containing graphite and metal oxide is located in a furnace at  $1200^\circ\text{C}$  [88]. By irradiation of 532 nm laser onto the composite, EMFs and empty fullerenes are generated and flow through the tube with inert gas and then they deposit on the tube wall [73,89]. Nonetheless, this method is expensive and the production rate is really low, so the large-scale production of EMFs with this method is limited. Contrary to laser ablation technique, the contact arc developed by Kratschmer and Huffman could be applied for mass production of EMFs [2,69,71]. A schematic image of its process is shown in Fig. 6.

Inhere, metal/graphite composite rods are used as anode and are exposed to a temperature above  $1600^\circ\text{C}$  in order to form metal carbides in the composite rod for efficient production of EMFs. Then, composite rods are arced in direct current in the range of 300–500 A and under flowing of He as a cooling gas. In the further step, the produced soot can be collected [90]. This method is

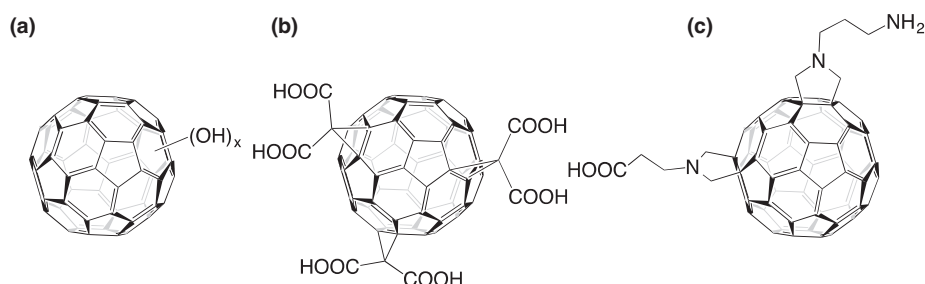


FIGURE 5

Structures of: (a) polyhydroxylated fullerene; (b) carboxyfullerene; (c) amino acidic fullerene.

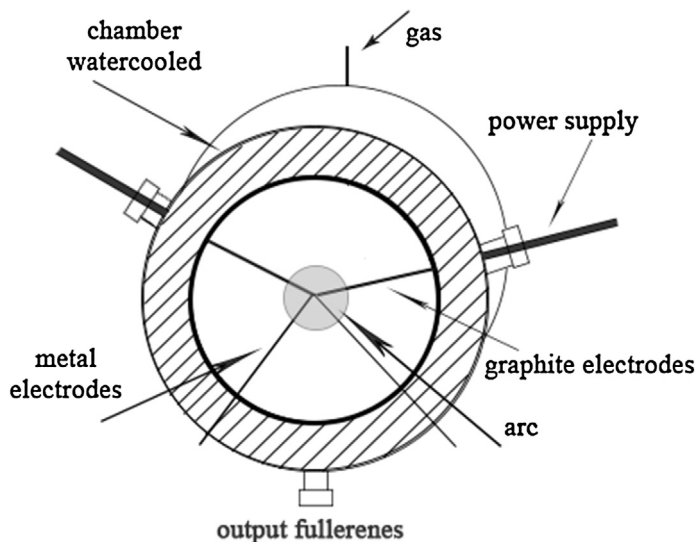


FIGURE 6

Schematic view of a Krätschmer-Huffman generator for production of endohedral metallofullerenes.

proficient and cost-effective for production of EMFs [87]. There are two main obstacles in the extensive research regarding the structural properties of EMFs. First, the difficulties in separation of EMFs culminated in the low availability of pure and symmetric single crystal of endohedral metallofullerenes. Second, because of the intrinsic features of EMFs, investigation of them with standard tools faced with numerous limitations [87]. In addition to low amount of single crystals of endohedral metallofullerenes, X-ray diffraction investigations of single crystals were impeded rotational disorder of the single crystal of fullerene molecule [87].

#### Fullerene-based nanomaterials

Self-assembly is a technique which has been used for synthesizing functional molecules with a variety of nanostructures. Nano-sized fullerene crystals have intermediate size, between molecules and bulk materials. Various methods exploited this characteristic in order to prepare 1D or 2D fullerene-based nanostructure [91].

Self-assembled fullerene nanostructure with disparate shapes could be synthesized by vapor-driven crystallization, liquid-liquid interfacial precipitation (LLIP), drop drying process and template assisted dip drying [92]. Fullerene fine fibers, which contain single-crystal  $C_{60}$ , present diameters ranging from 100 nm to a few microns and their lengths are hundreds of microns. They are

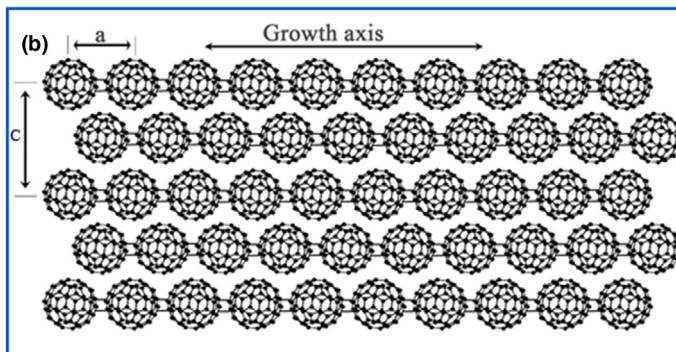
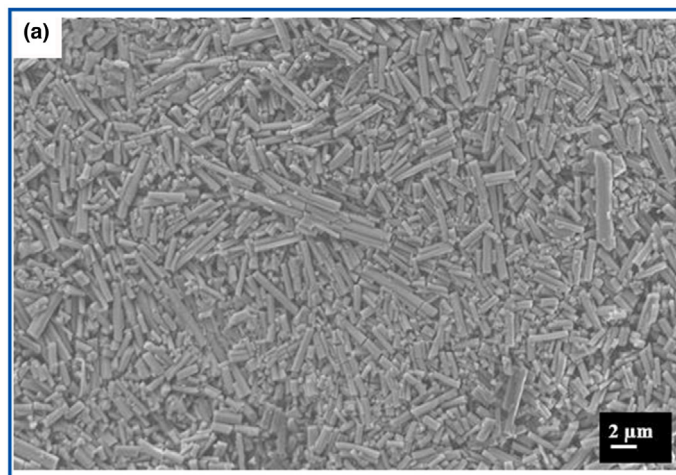


FIGURE 7

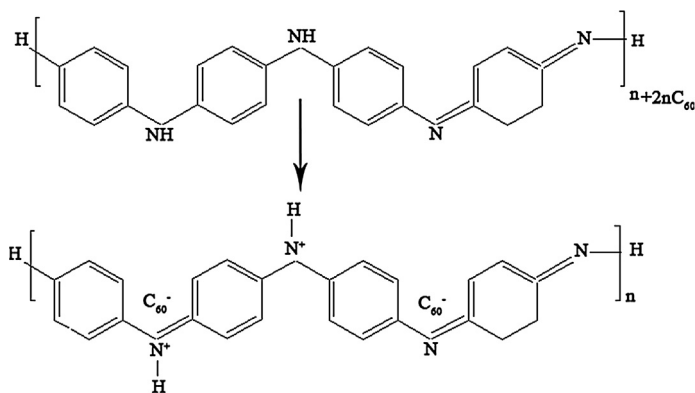
(a) SEM image of fullerene nano whiskers showing the morphology of the nanowiskers [97]; (b) model of one-dimensional fullerene nanowiskers with body-centered tetragonal crystal structure where  $a$  and  $c$  are lattice constants relative to the growth axis.

called  $C_{60}$  nanowiskers, and represent a 1D fullerene nanostructure (Fig. 7a) [91,93]. Fullerene nanowiskers are crystalline thin fibers and can contain pristine  $C_{60}$ , or/and endohedral or/and functionalized fullerenes [93]. The nanowiskers could be synthesized by liquid-liquid interfacial precipitation (LLIP) method, which is the most convenient procedure for preparation of Fullerene nanowiskers and it was developed by Miyazawa and colleagues [94-96]. In this method a solvent, in which fullerene presents low solubility as ethanol, isopropyl alcohol (IPA) and isobutyl alcohol, is mixed with a solvent able to properly dissolve  $C_{60}$  as toluene,  $m$ -xylene, benzene, and  $CCl_4$ . The liquid-liquid interface

between these two types solvents is the nucleation site for fullerene crystals [93]. The increase in fullerene solubility in one of the solvents will enhance the liquid–liquid interfacial pressure which is required for polymerization of fullerene molecules [97]. Fullerene nanowhiskers with lengths in hundreds microns and diameter in several nanometers possess a high aspect ratio and also a Young's modulus higher than pristine  $C_{60}$  [97]. The schematic model of a body-centered tetragonal crystal fullerene nanowhiskers and its growth direction is shown in Fig. 7b.

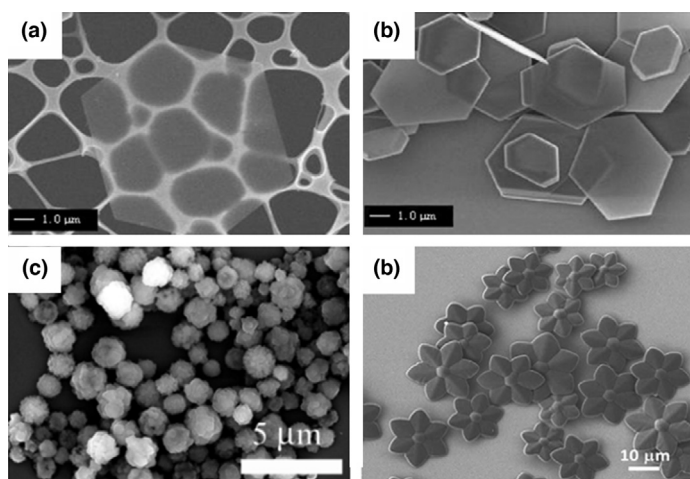
In order to improve the characteristics of fullerene nanowhiskers and prepare FW/PAni hybrid material, polyaniline emeraldine base dissolved in *N*-methyl-2-pyrrolidone (PAni/NMP) was added to fullerene nanowhiskers by direct-mixing technique. Despite fullerene nanowhiskers, this hybrid material has a tubular structure with a pleated surface morphology and consequently has a higher aspect ratio. The possible mechanism for the formation of this tubular structure (Fig. 8) is charge-transfer (CT) complex between the electron-rich polyaniline to electron-deficient fullerene nanowhiskers [98]. In another study,  $C_{60}$  nanowhiskers have been prepared by LLIP method and vortex-flow-induced alignment method has been used for alignment of  $C_{60}$  nanowhiskers. In this method, mechanical stirring of the medium induces a vortex flow which aligns fullerene nano whiskers [99]. The aligned  $C_{60}$ NWs on a glass substrate were used as a scaffold for the culture of human osteoblast MG63 cells. The results of study demonstrated that cell growth orientation was compatible with axis of  $C_{60}$ NWs and these nanowhiskers were a significant substrate for adherence and growth of MG63 cells [99].

In addition to fullerene nanowhiskers, recently, fullerene cylindrical nanotubes with improved semiconducting and electronic properties were synthesized via LLIP method. In this procedure *m*-xylene and TBA (tetra butyl alcoholic) were used as a saturating solvent and precipitation agent respectively [100]. In 2007, Sathish *et al.* [101] synthesized a flexible, porous and transparent fullerene hexagonal thin crystalline nanosheets via LLIP method, using alcohol and  $CCl_4$ . In this technique, the number of carbon atoms and the polarity of alcohols influence the particle size of the supramolecular structure, and high polarity alcohols culminate in the formation of uniform hexagonal nanosheets, the SEM image of which is shown in Fig. 9a. However, despite these indications, it is really hard to obtain a nanostructure with a certain morphology



**FIGURE 8**

The possible mechanism for the intermolecular interaction between fullerene nanowhiskers and imine group of PAni.



**FIGURE 9**

(a) SEM images of fullerene nanosheets; (b)  $C_{60}$ /Fc nanosheets [102]; (c)  $C_{60}$  Konpeito-like nano crystals at the interface of IPA and saturated solution of  $C_{60}$  in EB with  $C_{14}G_2$  surfactant [104]; (d) flower-shaped fullerene crystals [105].

in self-assembly of fullerene because of the presence of many influential factors [101].

A hybrid hexagonal nanosheet containing  $C_{60}$  and ferrocene has been prepared via LLIP method. The SEM images of these nanosheets can be observed in Fig. 9b [102]. The presence of high charge-transfer (CT) band between fullerene and ferrocene demonstrates that donor–acceptor interaction exists in the nanosheets and encourages the formation of  $C_{60}$ /Fc nanosheets [102]. Recently, pressure induced transformation of ferrocene-doped  $C_{60}$  nanosheets was investigated by *in situ* Raman spectroscopy in order to examine their controlled polymerization and form new planar polymers. With increasing the pressure, orientation and polymerization of  $C_{60}$  layers happened. The presence of light irradiation in this polymerization process makes it a more controlled process and increases the formation of new layers [103].

In 2015, Shrestha *et al.* [106] reported the synthesis of a assemble fullerene nanocrystal at liquid–liquid interface of  $C_{60}$  solution in butylbenzene and IPA. Low solubility of  $C_{60}$  in IPA and slow diffusion of IPA to fullerene promotes the nucleation of small fullerene clusters and further growth of crystals in hexagonal bipyramid and flower-like shape at liquid–liquid interface [106]. Later, the same group investigated the effect of natural nonionic surfactants, diglycerol monomyristate ( $C_{14}G_{12}$ ) and diglycerol monolaurate ( $C_{12}G_2$ ) on crystallization of  $C_{60}$  and the morphology of fullerene crystals [104], based on their report, the applied surfactants caused to a morphological transition in fullerene crystals from a 1D faceted rod to a unique zero dimensional nanostructure called ‘konpeito-like’ fullerene crystals (Fig. 9c). While in the absence of surfactants the ultimate morphology was the one dimensional nanorod with hexagonal close-pack structure [104].

One of the most recent fullerene crystal morphologies is fullerene flower which has been prepared via solution-phase crystallization of fullerene molecules. The presence of mesitylene as a good solvent, ethanol as a poor solvent and  $C_{60}$  and  $C_{70}$  molecules as the solute and the different between  $C_{60}$  and  $C_{70}$  solubility as the main factor for occurrence of a two-step crystallization resulted in a unique nucleation process and formation of the flower-shaped

crystals [105]. By controlling the crystallization procedure and changing effective factors, different fullerene flowers can be obtained. The SEM image of fullerene flower crystals is shown in Fig. 9d [105].

Another group of fullerene nanomaterials are polymer/fullerene nanocomposites which can present novel physico-chemical properties. Numerous polymers such as polystyrene (PS) and polyethylene glycol have been used as matrices for these nanocomposites [107]. Li *et al.* [108] in 2000 produced a nanocomposite including fullerene and polycaprolactone (PC) and improved electron accepting characteristics of the nanocomposites. They integrated fullerene derivative 1,2-(1',1',2',2'-tetracyanomethano)C<sub>60</sub> (TCNEO-C<sub>60</sub>) into polycarbonate (PC), the structure of which is shown in Fig. 10a, moreover they covalently linked N-methyl-pyrrolidino[60]fullerene to polycarbonate chain (MPYLD-C<sub>60</sub>-PC, Fig. 10b).

In 2007, a nanocomposite containing fullerene and polystyrene-polyisoprene-polystyrene (SIS) and polystyrene-polybutadiene-polystyrene (SBS) copolymers was synthesized (Fig. 11) [109] and their characteristics were investigated, finding that with the increase in fullerene content, the nano-tack adhesive forces decrease and the bulk forces also showed results similar to nanoforces.

Another nanocomposite containing fullerene was synthesized by Ag nanoparticles embedded in C<sub>60</sub> matrix via thermal co-deposition and the effect of temperature and nanoparticles percent in silver-fullerene nanocomposites was examined [110].

Li *et al.* [111] developed a polymer/fullerene nanostructure with defined morphology and polymer fiber orientation via a novel technique based on non-covalent interactions between copolymer and fullerene derivative. Another group of fullerene nanocomposites are gel-nanocomposites, in which fullerenes are embedded in the fibrillary gel networks. Incorporation of fullerene boosts the stability of the gels and their melting point. One example of these composites is fullerene-PEG gel, which has acceptable thermal stability and solubility in solvents like methanol, water and THF [112].

Another group of polymer/fullerene nanostructure is polymer/fullerene core-shell composite nanofibers (NFs) which can be applied in solar cells and can enhance the performance of polymer based solar cells [113]. A novel composite containing conjugated polymer (poly[2-methoxy-5-(2-ethylhexyl-oxy)-p-phenylenevinylene], MEH-PPV) nanoparticles and fullerene phenyl-C<sub>61</sub>-butyric acid methyl ester (PCBM) has been developed for applications in photodynamic therapy (PDT) in which MEH-PPV/PCBM nanoparticles generate ROS (reactive oxygen species) and exert toxicity

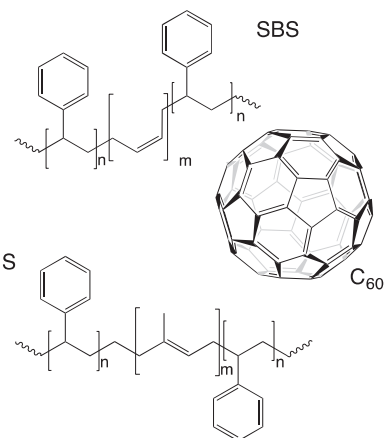


FIGURE 11

Chemical structure of SBS, [60]Fullerene and SIS.

towards cancerous cells [114]. Conjugation of glycol chitosan (GC) grafted with 2,3-dimethylmaleic acid (DMA) and C<sub>60</sub> (GC-g-DMA-g-C<sub>60</sub>) is a self-assembled polysaccharide nanogel containing hydrophobic (C<sub>60</sub>) and hydrophilic (GC and DMA) parts that can be used as a photosensitizer prodrug in PDT [115].

Fullerene hybrid macromolecular structures were synthesized by aggregation of C<sub>60</sub> and terpolymer colloids in isopropyl alcohol and the mixture of isopropyl alcohol-water. The results demonstrated that fullerene macromolecular hybrid nanostructures in isopropyl alcohol were spherical structure with uneven surface, while in isopropyl alcohol-water mixture clusters of crystalline nature of fullerene were observed [116]. Recently, a group reported the preparation of a nanostructure containing fullerene C<sub>60</sub> and carbon composite film via thermal sublimation of in a vacuum of C<sub>60</sub> powder and then the films were deposited onto substrate materials [117].

The development of more fullerene based nanostructures provides the opportunities for enhancement of fullerene applications in various fields like biomedical science and can make fullerene a more biocompatible and applicable material.

#### Fullerene containing polymers (polyfullerenes)

Since immediately after the fullerene discovery, fullerene-containing polymer emerged in materials world being able to associate extensive and favorable properties of polymers with exceptional characteristics of fullerenes [118]. Therefore, many efforts allocated to the synthesis of fullerene-containing polymers or polyfullerenes and a large number of publications reviewed those materials. Polyfullerenes can be classified into certain groups including all-C<sub>60</sub> polymers, cross-linked polymers, organometallic polymers, C<sub>60</sub> dendrimers, end-capped polymers, star shaped polymers, main chain polymers, side chain polymers and supramolecular polymers [119]. All-C<sub>60</sub> polymers also named 'intrinsic polymers' can be synthesized via covalent linkage of fullerene units [120]. Organometallic fullerenes can be considered as a kind of all-C<sub>60</sub> polymers which contain a metal or element in their structures [121]. The most favorable properties were attained via palladium copolymerization [122]. Cross-linked polymers are a group of polyfullerenes, which can be synthesized by random reactions with assistance of C<sub>60</sub>. Preparation of these cross-linked C<sub>60</sub>-containing polymers can be performed by reaction of polymers with C<sub>60</sub>

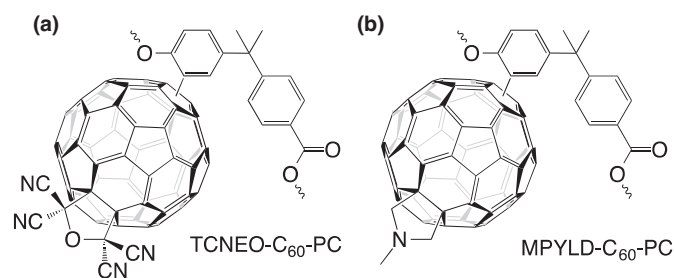


FIGURE 10

Polycarbonate containing fullerene derivatives.

randomly and in three dimensions [123]. Another class of fullerene-containing polymers entitled fullerene dendrimers have a regular branches and at least one fullerene molecule. Fullerene molecules can be located at the center, at the surface or in the connection points of the dendrimer [124,125].

The end-capped polymers are considered as a fascinating group of polyfullerenes, which possess one or two fullerene molecules placed on the end of polymer backbone and alter its properties. Fullerene units can be added after the polymer synthesis or, otherwise the growth of the polymer chain can started from the fullerene molecule itself [119]. Star-shaped polymers containing a fullerene unit present from two to twelve long, flexible chains with a topology similar to sea-stars. These polyfullerenes can be prepared with two approaches: (a) polymer chains can be linked to fullerene unit, or (b) polymer chains can be grown from the surface of fullerene unit [126,127].

Another class of  $C_{60}$ -containing polymers is the one in which fullerene units are placed in the backbone of a polymer and formed a structure similar to a necklace. This polyfullerene can be synthesized by direct reaction of difunctionalized monomers and fullerene cage and also by polycondensation between a fullerene bisadduct and difunctionalized monomers [128,129]. The most studied group of  $C_{60}$ -containing polymers is side-chain polymers. These polymers can be prepared in two ways, by direct reaction of fullerene units with preformed polymers or by functionalization of fullerene in order to be able to copolymerization with other monomers [130,131].

The last class of  $C_{60}$ -polymers is represented by supramolecular polymers prepared by Dai *et al.* [132] in 1999. According to the synthetic method, supramolecular fullerenes are divided into four types, (a) interaction between fullerene and functionalized polymers; (b) fullerene derivative self-assembly; (c) multifunctionalization of  $C_{60}$  and complementary polymeric backbones; and (d) supplementary interactions between pristine fullerene and ditopic concave guests.

### Toxicity and biocompatibility evaluation of fullerenes

Since pristine and derivatized  $C_{60}$  can access to intracellular space and accumulate into cell membrane and cytoplasm, they might be a threat for cell functioning and integrity [133–139]. Physicochemical properties, ROS-related behavior and toxicity of fullerene can generate different results depending on the techniques employed for the solubilization of pristine  $C_{60}$ , including mechanical processing, long term stirring in water, and chemical modifications that can change the general properties of pristine fullerene [140].

#### *In vitro* toxicity of pristine and derivatized fullerene

The toxic effects of fullerene colloid prepared with tetrahydrofuran (THF/ $nC_{60}$ ) on different mammalian cells have been proven in a number of studies, which demonstrated ROS production, mitochondrial depolarization, and lipid peroxidation resulting to necrotic cell death [141–143]. One investigation to determine how THF affects fullerene toxicity *in vitro* has been done by Kovochich *et al.* in 2009 [144]. The authors found that, in mouse macrophage cell line, the supernatant of THF/ $nC_{60}$  induces toxicity rather fullerene aggregates because of the presence of THF byproducts including  $\gamma$ -butyrolactone and formic acid. In fact, these byproducts cause intracellular  $Ca^{2+}$  release,  $Ca^{2+}$

increase in mitochondria and their perturbation. Therefore, the toxicity of THF/ $nC_{60}$  seems to be related to the method used for fullerene preparation rather than to the intrinsic properties of pristine fullerene [142–145]. Another study also showed that THF peroxide is the main factor in oxidative reactivity of THF/ $nC_{60}$  and also this THF derivative plays a significant role in antibacterial activity of THF/ $nC_{60}$  towards *Escherichia coli* [146]. In further studies, it has been confirmed that neither fullerene nor THF are responsible for toxicity, and indeed THF byproducts, as  $\gamma$ -butyrolactone and THF hydroperoxide, induced toxicity, so extra washing steps to remove these byproducts led to absence of toxicity of THF/ $nC_{60}$  in *D. magna* and A549 lung cells [147]. Further studies showed that preparation of  $C_{60}$  nanoparticles obtained by solvent exchange using ethanol or toluene instead of THF, as well as water-stirred  $C_{60}$  suspensions did not cause cell damage, these finding favored the statement mentioned above [143,148]. Markovic *et al.* [140] in 2008 prepared aqu/ $nC_{60}$  via long term stirring in water, which had low fullerene concentration and yielded large aggregates formation. The aqu/ $nC_{60}$ , made by sonication, willingly produced singlet oxygen upon illumination [149], although according to other studies it was not photoactive [150]. In ambient light situations, aqu/ $nC_{60}$  did not produce reactive oxygen species and showed only marginal ROS-independent cytotoxicity in diverse mammalian cells [143]. It seems that the fullerene functionalization degree can affect its tendency to form aggregate and monofunctionalized molecules have more tendency to be aggregated that polyfunctionalized fullerenes, which is more stable in solution [151]. Commonly, there is a belief that derivatization of fullerene can induce considerable decrease in toxicity for the functionalized fullerenes. Therefore, highly soluble fullerenes have less cytotoxic activity since the increase in the number of functional groups covalently attached reduces the ROS-generating capacity in fullerenes [141,152]. Polyhydroxylated fullerenes (named fullerols or fullerlenols), when irradiated with UV and visible light, produce singlet oxygen, although fullerlenols produce less ROS than polymer coated fullerenes, they induce photooxidative stress in human cells [153,154]. However, at sub-cytotoxic concentration fullerlenols are able to scavenge hydroxyl radicals [155]. Similarly to fullerlenols, carboxyfullerenes are antioxidative/cytoprotective agents [156] that, under light irradiation, exhibit cytotoxicity.

As mentioned, the ability of fullerene to penetrate cell membranes extensively influences its toxicity: cationic fullerene derivatives that can easily enter cells are more toxic than neutral and anionic fullerene derivatives [157]. Parental administration of fullerenes in diagnostic and therapeutic applications, pulmonary exposure and inhalation of fullerene nanoparticles may lead to cardiovascular adverse effects, therefore, a number of studies evaluated fullerene cardiovascular toxicity [158,159]. While  $C_{60}$  preparations obtained by sonication elicited platelet aggregation in a lower extent than other carbon nanoparticles, not developing thrombosis, the organic solvent-prepared  $C_{60}$  was found to adhere to platelets, which may result in thrombosis [68,160].

As previously mentioned, the water-stirred  $C_{60}$  and fullerlenols were found to induce intracellular  $Ca^{2+}$  increase and cause arrest G1 cell cycle phase in human umbilical endothelial cells, although in the case of water-stirred  $C_{60}$  no cell death was observed, while fullerlenols cause apoptotic cell death, which may encourage



coronary artery diseases and atherosclerosis [159]. In another study it was shown that fullerenols caused accumulation of polyubiquitinated proteins and autophagy, leading to a decrease in cell density [161]. In contrast with other fullerene nanoparticles and derivatives, hexasulfobutyl- $C_{60}$  is able to inhibit low-density lipoprotein (LDL) from oxidation, so it can be effective in atherosclerosis prevention [162].

In addition to cardiovascular toxicity, the blood compatibility of fullerene *in vitro* has been investigated in a number of studies. THF/ $C_{60}$  causes oxidative stress mediated lysis of erythrocyte cells associated with shrinkage and loss of their normal shape, while no intentional photoirradiation was applied [163]. When fullerenes enter the blood stream they will translocate to liver, therefore investigation of toxic effects of fullerenes on liver seems to be important.  $\gamma$ -Cyclodextrin/ $C_{60}$  upon photosensitization triggers singlet oxygen-dependent oxidative stress in rat liver microsome, inducing protein oxidation and lipid peroxidation [164]. The results obtained using polyhydroxylated fullerene were similar to  $\gamma$ -cyclodextrin/ $C_{60}$  [165].

Further studies showed toxic effects of  $C_{60}(OH)_{24}$  under ambient light on rat hepatocytes which induced lipid peroxidation and depletion of intracellular ATP, leading to cell death, while  $C_{60}(OH)_{12}$  exhibited less toxic effects. These results demonstrated that the toxicity effects of fullerenols are dependent on the number of hydroxyl groups present on the fullerene carbon cage, and probably to their solubility too [166].

Another cell type which has been tested for THF/ $C_{60}$  toxicity are canine kidney cells [167], and also mouse renal epithelial cells were used to determine some toxicity of sonicated  $C_{60}$ , which decreases the transepithelial electrical resistance, an indicator of epithelial barrier function [168]. Polyhydroxylated fullerene caused cytoskeleton disruption, mitochondrial dysfunction followed by ATP depletion [169]. The applications of fullerenes as drug carriers in order to bypass blood ocular barriers nudge researchers towards assessment of ocular toxicity of fullerene and its derivatives. Upon UV irradiation  $\gamma$ -cyclodextrin/ $C_{60}$  inducing oxygen-mediated protein peroxidation which led to apoptotic cell death, while in the dark or ambient light no toxic effect was observed [170]. The more aggregated  $\gamma$ -cyclodextrin/ $C_{60}$ , the less singlet oxygen production, therefore fully aggregated fullerene showed no toxicity effect on lens cells. In investigation of fullerene toxicity, it has been observed that lower fullerene concentrations caused oxidative damage to human lens epithelial cells under UV and visible light irradiation, while higher concentrations showed toxicity even in the dark [171]. Another study reported that the toxic effect of fullerene on retinal pigment epithelial cells was similar to its toxicity to human lens epithelial cells [172], indicating that the use of fullerenols in ocular application, especially in the presence of sunlight, may result in lens or/and retinal damage [173].

Solvent exchange prepared THF/ $C_{60}$  nanoparticles and also polyvinyl pyrrolidone/ $C_{60}$  complexes can be immediately internalized by human keratinocytes and then accumulated in cytoplasm near the nucleus [174,175]. It has been shown that THF/ $C_{60}$  nanoparticles decreased the growth of human keratinocytes and under the light its effect increased somewhat [176]. However, further studies reported the toxic effect of THF/ $C_{60}$  nanoparticles on human keratinocytes and also human dermal fibroblasts [141,143].

The complexes of  $C_{60}$  with  $\gamma$ -cyclodextrin, sodium dodecyl sulfate, or polyvinyl pyrrolidone prepared by mechanochemical approach have no cytotoxicity *in vitro* [143]. In addition they can reduce ROS generation, melanin production in human keratinocytes and melanocytes under UV irradiation [175,177,178]; this characteristic makes them applicable in skincare products [179], though,  $\gamma$ -cyclodextrin/ $C_{60}$  under UVA irradiation caused oxidative stress in keratinocytes [180]. In contrast reports related to cytotoxic evaluation of polyhydroxylated fullerene, it has been found that  $C_{60}(OH)_{32}$ , unlike  $C_{60}(OH)_{24}$  and  $C_{60}(OH)_{20}$ , in the absence of light killed human keratinocytes [181], while other studies showed that, in human keratinocytes, polyhydroxylated fullerenes blocked DNA damage and oxidative stress induced by UV irradiation, so the increase of hydroxyl groups elevated the cytoprotective activity [178,182].

The reported results demonstrated that both antioxidant activity and toxicity in the dark rise with the increase in functionalization groups attached to fullerene cage, even though, in general, the definitive effect probably depends on the concentration [173]. Polymer coated fullerenes, in the absence of overt light irradiation, exhibited no toxicity towards neuronal cell lines [183,184]. The carboxylated fullerene and hydroxylated fullerene also showed no toxicity to neural cell lines; however, they decreased the viability of primary mouse neurons [185].

Recently, an investigation has been accomplished in order to study the cytotoxicity of two type fullerene solutions on Chinese-hamster V-79 cells [186]. Two fullerene solutions, one prepared via solvent exchange method and the other prepared via N-methylpyrrolidone (NMP), in which cluster size is smaller, were prepared and tested, and in both cases no toxicity was reported in *in vitro* tests, demonstrating that N-methylpyrrolidone can be exploited as solvent in preparing aqueous fullerene solutions [186].

#### *In vivo toxicity of pristine fullerene and derivatized fullerene*

Since nanoparticles are able to enter systemic circulation and exhibit systemic and local toxicity, *in vivo* evaluation of their toxicity seems to be significant. Moreover, *in vivo* tests can demonstrate fullerene toxicity more accurately than *in vitro*, as this approach simulates the body environment in more satisfactory way. The first *in vivo* study was accomplished in 1996 by injecting an aqueous suspension of micronized  $C_{60}$  to rodents. In these experiments,  $C_{60}$  demonstrated to be no deadly, and did not show acute or sub-acute toxicity to this animal species [187,188].

Fullerenes are able to localize in lipid-rich regions like cell membranes based on *in vitro* experiments, and they are redox active. Additionally, as fullerene can form colloidal aqueous solution, the oxidative damage caused by fullerene has been studied in aquatic species, as bass [189]. The results of this study have shown that, despite change in protein oxidation in any tissue, changes were observed in lipid peroxidation, in gill and liver lipid peroxidation, with their decrease, while, in contrast, brain showed a trend of increased lipid peroxidation. These differences might be attributed to superior antioxidant defenses of gill and liver with respect to brain tissues, or fullerenes might reach the brain before the dissociation of the colloids. Although this selective localization of fullerenes in cell membrane is fascinating, a balance between advantageous therapeutic and potential toxic effects can be avoided by coating the fullerenes. Another

study investigated the effect of the THF of entrapped into fullerene aggregates on their toxicity in larval zebrafish [190]. THF have been used to prepare aqueous C<sub>60</sub> aggregates [25], therefore it is possible that the ascribed toxicity of fullerene aggregates [189] is a result of the presence of THF in the preparation [190]. The results demonstrated that THF-C<sub>60</sub> and THF-water induced change in gene expression and survival of zebrafish larvae, while C<sub>60</sub>-water had no effect on fish mortality. Although GC-MS did not detect THF in water sample, byproducts generated from THF treatment, as the already mentioned  $\gamma$ -butyrolactone, seem to be responsible for the toxic effects [190]. Also the changes in gene expressions seem to be related to THF and/or its degradation products in fact C<sub>60</sub>-water treatment did not induce gene expression changes in zebrafish, although longer exposure to C<sub>60</sub> might lead to different result [190].

Further studies have been performed on the animal exposure to atmosphere containing fullerene, in order to investigate the effects of fullerene existing in urban atmospheres. In mice treated by intratracheal instillation of pure sonicated C<sub>60</sub>, fullerenes aggregated have been found in alveolar and capillary lumen and also in pulmonary lymph nodes after exposure [191]. Though no lung inflammation or histopathological abnormalities were observed, the presence of fullerene at the air-blood barriers, 5 min after exposure, suggests that inhalation of fullerene resulted in rapid translocation of C<sub>60</sub> nanoparticles through systemic blood circulation followed by spreading in diverse tissues in the body. In 2009 Fujia *et al.* exposed rats to inhalation of surfactant (Tween 80)-dissolved pristine C<sub>60</sub> [192]. After 4 weeks, fullerene particles have been seen in alveolar macrophages; however, no lung tissue abnormality has been observed. In other studies, intratracheal instillation of fullerene at high dose (3 mg/kg) significantly increased the neutrophil-rich inflammation areas up to 1 week after the administration and also caused a transient increase in the number of neutrophils in bronchoalveolar fluid [193,194].

According to these results, C<sub>60</sub> nanoparticles induce only transient lung inflammation and no pathologic changes in lung and also other tissues. Sayes *et al.*, in 2007, observed no increase in lactate dehydrogenase, alkaline phosphatase and protein values in bronchoalveolar fluid up to 3 month after the rat intratracheal instillation of THF/C<sub>60</sub> prepared by solvent exchange [195]. According to the results reported by various studies investigating pulmonary toxicity of pristine and functionalized (hydroxylated) fullerene, their instillation/inhalation can cause transient inflammation in rats and mice, though continuous inflammation followed by tissue damage was observed at high doses [173]. After intraperitoneal injection of Tween-solubilized C<sub>60</sub> (500 mg/kg), fullerene accumulated in liver, especially in Kupffer cells and rarely in hepatic stellate cells and hepatocytes, and no parenchymal cell damage, inflammation or fibrosis were observed with microscopic examination. In addition, C<sub>60</sub> aqueous solution preserved liver from ROS mediated toxicity of CCl<sub>4</sub> [196]. In another study in rats performed with THF/nC<sub>60</sub>, the injected fullerene accumulated in liver, heart, kidney, spleen, lung, intestine, and muscle and bone tissues. The dose of 0.25  $\mu$ g/kg of nC<sub>60</sub> was not toxic and no symptoms were observed after two weeks of injections; this finding was in contrast with *in vitro* cytotoxicity of THF/nC<sub>60</sub> on melanoma cells [197]. Administration of polyalkylsulfonated C<sub>60</sub> to rats caused diffuse necrosis of tubular epithelium in kidneys, moreover

some pigment-laden macrophages were observed in spleen, liver and thymus [198].

Shinohara *et al.* examined genotoxicity of fullerene by *in vitro* and *in vivo* experiments [199]. They synthesized C<sub>60</sub> nanoparticle suspensions in order to test chromosomal aberration and bacterial reverse mutation under light irradiation and in dark condition. Eventually, they concluded that C<sub>60</sub> nanoparticle suspensions do not have genotoxicity neither in the dark nor under light irradiation [199].

A study on fullerene toxicity was performed by Yamago *et al.* [200]: by a single intra-peritoneal injection of a water-soluble methanofullerene (0.5 g/kg of bodyweight), the mice survived for 1 week. After single intraperitoneal injection of polyhydroxylated C<sub>60</sub> to mice (1.2 g/kg), the activity of cytochrome P450-dependent liver monooxygenase was reduced by the inhibition of the enzyme catalytic activity. Lower doses of fullerenol injected intraperitoneal did not influence blood cells in rats; however, antioxidative capacity of erythrocytes was reduced [201]. By using lower dose of fullerenol (50 mg/kg), toxic effects completely disappeared [202,203].

Through intravenous injection to rats, THF/nC<sub>60</sub> was delivered all over the body with a high rate of uptake for liver and spleen [204]. After injection of fullerene aqueous suspension, the molecule rapidly disappeared from circulation and accumulated in the liver and it was also observed in muscle, lung and spleen, but not in the brain [176].

In order to evaluate fullerenol as a photodynamic therapy agent, it has been injected to mouse and, similar to pristine C<sub>60</sub>, it was rapidly cleared from circulation and accumulated in liver, lung, and spleen. Moreover, the results demonstrated that fullerenol uptake by tumor cells was higher than normal tissue and so it can be used as photosensitizer in photodynamic therapy of some types of tumors [205].

A fullerene ammonium salt is also rapidly cleared from circulation; however, its rate is lower than pristine C<sub>60</sub>. Most of it accumulated in the liver and the rest in lung, muscle, and skin. Similarly to pristine C<sub>60</sub>, it did not reach the brain [176]. The result of intravenous injection of polyalkylsulfonated C<sub>60</sub> (100 mg/kg) was similar to its intraperitoneal injection and it caused phagolysosomal nephropathy in rats [198].

In addition to systemic administration of fullerene, a number of studies investigated oral administration of fullerene. A single dose of (Tween 80)-solubilized C<sub>60</sub> (2 g/kg) has orally administered to rats and no sign of toxicity or abnormalities in any tissue was observed [206]. In another investigation, oral administration of C<sub>60</sub>, dissolved in olive oil, not only does not induce chronic toxicity, but also doubles the lifespan of rats [207]. In contrast with these findings, an orally administrated lower dose of pristine C<sub>60</sub> (0.64 mg/kg) in corn oil caused DNA damage in liver and lungs [208]. From these studies it emerged that the gastrointestinal adsorption of fullerene and its consequent toxicity is low; however, in a number of experiments oxidative damage of DNA mediated by pristine C<sub>60</sub> has been reported and this deserves substantial consideration [173].

Also dermal toxicity of fullerene deserves special attention due to the cosmetic application of fullerene relating to its antioxidant activity [173]. Xia *et al.* [148] in 2010 revealed that penetration of C<sub>60</sub> on pig skin is depending on fullerene vehicle. For example,

penetration of chloroform was higher than toluene or cyclohexane, while C<sub>60</sub> in mineral oil did not penetrate skin. Investigation of C<sub>60</sub> in propylene glycol in rabbits, pigs and humans demonstrated no skin irritation or skin reaction [209]. In addition, polyvinyl pyrrolidone/C<sub>60</sub> showed no toxicity in under UV irradiation [210].

## Biomedical applications of fullerenes

### Antioxidant activity and radical scavenging

Free radicals are produced by normal cellular metabolism as well as abnormal reactions, encouraged by some disorders. They can trigger tissue abnormalities along with toxicities and disease processes [211], damaging biological molecules like proteins, lipids, and DNA. This leads to cell damage and, in some cases, to diseases such as cancer and atherosclerosis [212,213].

One of the main characteristics of C<sub>60</sub> is its exceptional free radical scavenging ability, which makes it favorable 'free radical sponge' in order to protect biological systems against cell damage and tissue abnormalities [37,38,213,214]. The presence of several double bonds in fullerene cage makes it able to react with free radicals [38,215].

Fullerenes, as the most efficient scavenger, can react with free radical species like superoxide (O<sub>2</sub><sup>•-</sup>), and hydroxyl radicals (OH<sup>•</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) without being consumed; moreover they can localize within the cells and inhibits free radicals production. On the other hand, based upon reported studies, it can be hypothesized that radical scavenging capability of fullerenes may not be entirely attributed to fullerene cage itself, but also it can related to functional groups attached to carbon cage, therefore, free radical deactivating capacity of various fullerenes is different [140,216]. Because of its insolubility in water, derivatized fullerenes are more favorable for radical scavenging; moreover derivatized fullerene can interact with cellular and subcellular environment more efficiently [140,217]. Wang *et al.* evaluated antioxidant activity of C<sub>60</sub>, of three of its derivatives and of vitamin E determining the inhibition of lipid peroxidation [218]. They reported that fullerene derivative efficiently can prevent peroxidation and membrane breakdown triggered by free radicals, and liposoluble fullerene derivative was more effective in inhibiting lipid peroxidation than vitamin E, which is a natural antioxidant. Their results also showed that both fullerene derivatives, liposoluble and water-soluble, can potentially be used as antioxidant derivatives. This property has been investigated also for other water-soluble fullerene derivatives, such as C<sub>60</sub>(OH)<sub>*m*</sub>, carboxyfullerenes, C<sub>60</sub>(ONO<sub>2</sub>)<sub>7±2</sub>, and hexa(sulfobutyl)fullerenes, finding good radical scavenging abilities [67,68,219–223].

Derivatization of fullerenes with polar groups like polyhydroxylated fullerenes and tris(malonic)acid-C<sub>60</sub> (TMA-C<sub>60</sub>) makes them able enter mitochondria, where free radicals are generated and also TMA-C<sub>60</sub> showed strong neuroprotection in several cell culture models of neurological diseases like Parkinson's [33,133].

Assessment of radical scavenging properties of aqueous fullerene suspension have been studied by Gharbi *et al.* [196] who demonstrated the prevention of free radical related damage in the liver. The antioxidant properties of dendrofullerene and TMA-C<sub>60</sub> derivatives have been investigated [224], together with amino acid derivatives of fullerene. For the latter case, fullerene bearing seven β-alanine demonstrated antioxidant properties against

hydrogen peroxide and prevented induced apoptotic cell death, since alanine-fullerene impeded ROS accumulation in extra- and intracellular environment. The α-alanine fullerene derivatives also showed efficient radical scavenging capability against superoxide and hydroxyl radicals [215,225]. Also another amino acidic fullerene derivative bearing five cysteines inhibits apoptosis induced by hydroxyl and superoxide free radicals [226].

Fulleropyrrolidines bearing one or two 3,5-di-*tert*-butyl-4-hydroxyphenyl units, reported by Enes *et al.* [227], have also confirmed the antioxidant properties. Lin *et al.* [228] investigated the antioxidant activity of carboxyfullerene by *in vitro* study and the iron-induced lipid peroxidation and auto-oxidation of brain homogenates were suppressed. Moreover, the *in vivo* studies showed that carboxyfullerene antioxidant activity preserves nigrostriatal dopaminergic system from iron-induced oxidative damage.

Another study investigated the antioxidant potential of carboxyfullerene for cells of immune system, with protection of inactive human peripheral blood mononuclear cells (PBMCs) from apoptosis induced by TNF-α plus cycloheximide or 2-deoxy-d-ribose (dRib) agents [229]. In summary, along with other biological applications, fullerenes can be used as powerful antioxidants and are more active than currently used compounds like vitamin E.

Yao *et al.* [230] investigated the radical scavenging kinetics of four fullerene polymeric systems including C<sub>60</sub>-PAA-C<sub>60</sub>, PAAC<sub>60</sub>, PDMA-C<sub>60</sub> and PEO-b-PAA-C<sub>60</sub>. The radical scavenging process of these fullerene polymeric systems contained two stage kinetic trends which relates to unimers and micelles existing in aqueous solution of fullerene polymeric system. Moreover, they reported that in free radical scavenging process micelles and unimers react with radicals. Increasing of the number of fullerenes in larger micellar cores results in faster reactions of radicals. In fact, they concluded that nanostructure has an effect on antioxidant activity of fullerene polymers [230].

### Cancer treatment and photodynamic therapy

As mentioned above, fullerenes have been known as a radical scavenger and antioxidant agents, but, paradoxically, they can act also as oxidants. In fact they are able to promote the production of ROS within cells and consequently can stimulate development of oxidative stress in some circumstances [231,232].

Prior to the hypothesis reported by Andrievsky *et al.* in 2009 [233], it was assumed that covalent bonds of fullerene react with ROS and form covalent bonds, however this assumption made difficult to explain how fullerenes produce ROS during photodynamic therapy [234]. Andrievsky *et al.* reported that hydrated fullerenes can inactive ROS by the ordered 'water coat' rather than covalently scavenging free radicals, considering that the ordered 'water coat' can trap two hydroxyl radicals for enough time to react with each other and generate less reactive hydrogen peroxide species [233].

One of the cancer treatment strategies using fullerenes is light-based therapies such as photodynamic therapy and photothermal therapy (PTT) [235,236]. Photodynamic therapy is a non-invasive and nonsurgical treatment for some type of tumors and some of non-malignant diseases. Photodynamic is a photochemical method that uses photosensitizing agents (photosensitizer [PS]) and

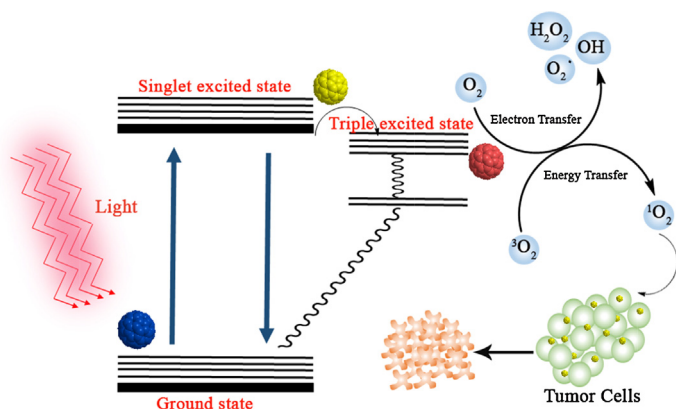
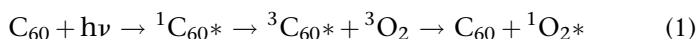


FIGURE 12

Schematic of photodynamic therapy with fullerene as photosensitizers.

light irradiation to produce reactive oxygen species in tumor tissue, to induce damage and apoptosis. The schematic of this process is shown in Fig. 12 [16,234,237].

Fullerenes can be applied as possible PS for photodynamic therapy [234,238]. Functionalization of the fullerene molecule can promote its capability to produce reactive species which can mediate PDT in medicinal applications [234]. When  $C_{60}$  is irradiated with visible light, it can be excited from  $S_0$  ground state to a short lived state,  $S_1$ , excited state.  $S_1$  quickly decay to lower lying triplet state  $T_1$  which has a long life time (50–100  $\mu$ s; Eq. (1)).



In the presence of dissolved oxygen ( ${}^3O_2$ ), existing as a triplet in its ground state, fullerene  $T_1$  is quenched to produce singlet oxygen ( ${}^1O_2$ ). So, pristine and functionalized fullerenes are able to catalyze ROS production upon light irradiation. *In vitro* studies for the evaluation of fullerene in photodynamic therapeutic applications were implemented in 1990s. Tokuyama *et al.* proved the phototoxic effect of fullerene functionalized with carboxylic acid on human cervical carcinoma cells [239]. The toxicity and superoxide production ability of water-soluble polyethylene glycol fullerene derivative under visible light was demonstrated by Nakajima *et al.* [240]. Burlaka *et al.* [241] also demonstrated the phototoxicity of pristine and functionalized fullerene in carcinoma cells by ROS generation: the phototoxicity of dendritic  $C_{60}$  monoadduct and TMA- $C_{60}$  derivatives was investigated on Jurkat cells (T-lymphocytes) under UV irradiation, and the results showed that tris-malonic acid derivative is more phototoxic than dendritic one in killing Jurkat cells [242]. Ji *et al.* also investigated the biodistribution and tumor uptake of  $C_{60}(OH)_x$  in five kinds of mouse bearing tumors demonstrating the utility of this derivative as photosensitizer in photodynamic therapy of some tumors [205].

One of the most soluble fullerene copolymers,  $C_{60}$ -N vinylpyrrolidone, under visible light irradiation demonstrated its potential as a photodynamic therapy agent [243]. Mroz *et al.* studied the photodynamic activity of cationic and hydrophilic functionalized fullerene towards mouse cancer cell lines [235]. They showed that the fulleropyrrolidinium salt could be used as an efficient photosensitizer for killing cancer cell by inducing apoptosis under illumination, with the already mentioned mechanism. The phototoxic activity of porphyrin- $C_{60}$  dyad (p- $C_{60}$ , its

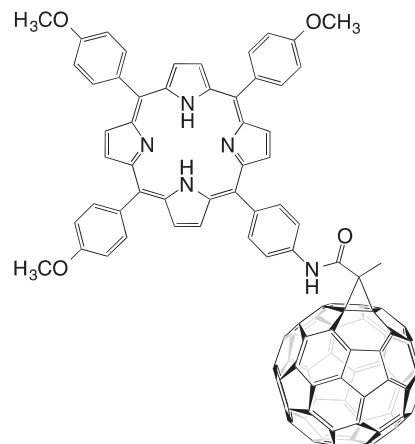


FIGURE 13

Molecular structure of PS porphyrin- $C_{60}$  dyad.

schematic shown in Fig. 13) on Hep-2 human larynx-carcinoma cells was assessed by Alvarez *et al.* [244]. The dyad has the potential to form photoinduced charge-separated state and can act as a photodynamic agent for cell inactivation. The mechanism by which P- $C_{60}$  induced apoptosis was caspase-3-dependent [244]. Zhao *et al.* evaluated the phototoxicity of four different forms of fullerene prepared by solvent exchange and using dispersant agent: the results showed all four preparation have potential in photogeneration of singlet oxygen and superoxide [180]. While in the dark no cytotoxicity was observed, the main photoinduced cytotoxic activity was due to singlet oxygen rather than superoxides [39]. The photodynamic activity of fullerene towards HeLa cells has been investigated, the combination of cucurbit[8]uril-fullerene complex and light resulted in HeLa cells death, due to damage of membrane proteins and phospholipids [245].  $C_{60}$ -PEG-Gd, which is prepared by mixing gadolinium acetate solution with  $C_{60}$ -PEG-DTPA, is a photosensitizer which have been developed by Liu *et al.* [246], the toxic effects and anti-tumor activity were evaluated by intravenous injection to mice bearing tumor and after light irradiation it resulted in photodynamic activity, irradiation time dependent.

*In vitro* experiments to evaluate the photodynamic therapeutic applications of pullulan-fullerene derivative showed that it can prevent the growth of HepG2 hepatoma cells, while experiments via intravenous injection exhibited stronger antitumor activity in comparison with  $C_{60}$ -PEG conjugate or saline [247]. Nobusawa *et al.* developed pH sensitive 6-amino- $\gamma$ -cyclodextrin (ACD) as carrier for  $C_{60}$  in order to release it for photodynamic activities on acidic surface of cancer cells [248]. *In vitro* experiments showed that ACD/ $C_{60}$  can be taken up into cancer cells. In the same year, Hu *et al.* [249] synthesized a fullerene derivative using L-phenylalanine, L-arginine and folic acid in order to alter fullerene limited solubility which leads to lower singlet production in photodynamic therapy. The results demonstrated that the uptake of this fullerene derivative in HeLa cells was higher than in normal cells and the subsequent photoirradiation raises  ${}^1O_2/O_2^{\bullet-}$  generation and induces cell apoptosis [249].

A newer approach for cancer treatment implies the combination of  $C_{60}$  derivatives with other molecules. For instance Shi *et al.* coupled iron oxide nanoparticles with  $C_{60}$ , and functionalized it with PEG, in order to improve its solubility [250]. This nanocomposite can be used for photodynamic therapy and MRI and also as a

drug delivery system if in combination an antitumor drug, hematoporphyrin monomethyl ether (HMME). In this case, the photodynamic effect is higher than for free HMME. Also a complex of C<sub>60</sub> with  $\gamma$ -cyclodextrin polymer has been synthesized in order to improve phototoxicity of fullerene and make it more water-soluble, with higher efficacy in producing singlet oxygen and killing cancer cells under UV irradiation [251].

Later, a drug delivery system including C<sub>60</sub>-PEI, a fullerene derivative loaded with doxorubicin (DOX, an anticancer drug) was developed to combine chemo- and photodynamic therapy for cancer treatment. The DOX release in this system is greatly dependent on pH values and in acidic condition (as in tumor cells) DOX was released with higher speed, with good inhibition of B16-F10 cells growth *in vitro* and, in *in vivo* experiment, good tumor suppression in murine melanoma cancer. Moreover, the side effects were lower than free DOX. The results gave credit to C<sub>60</sub>-PEI-DOX to be used as a nanomedicine for cancer treatment [252]. In other studies, C<sub>60</sub>-DOX combination was developed as antitumor complex [253,254]. At first Prylutskyy *et al.* examined the structural and physicochemical properties of fullerene-DOX complex for tumor inhibition [254], and in a later study [253], they compared antitumor activity of fullerene-DOX combination with pristine fullerene and DOX themselves, used separately, in the treatment of Lewis lung carcinoma in male mice. Based on their results, combination of fullerene and DOX was more efficient in inhibiting the tumor growth in *in vivo* models and it could be used as an effective approach in antitumor therapy [253].

Additionally, fullerene-DOX complex was investigated for its cytotoxic activity in tumor therapy [255], and the results revealed that presence of fullerene improved the cytotoxic effect of DOX towards tumor cells by increasing hydrogen peroxide cellular production although in *in vivo* model the effect was more noticeable than that in *in vitro* experiments [255]. These results are somewhat in contrast with a foregone study that has been carried out in 2005 [256], which demonstrated that fullereneol has modulating effects on three antitumor drugs (DOX, cisplatin, and taxol) when administered in combination. Further researches and investigations on C<sub>60</sub>-DOX complex have been developed and recently Prylutskyy *et al.* proved that C<sub>60</sub>-DOX hetero-complexation is responsible for their synergy in physiological media [257].

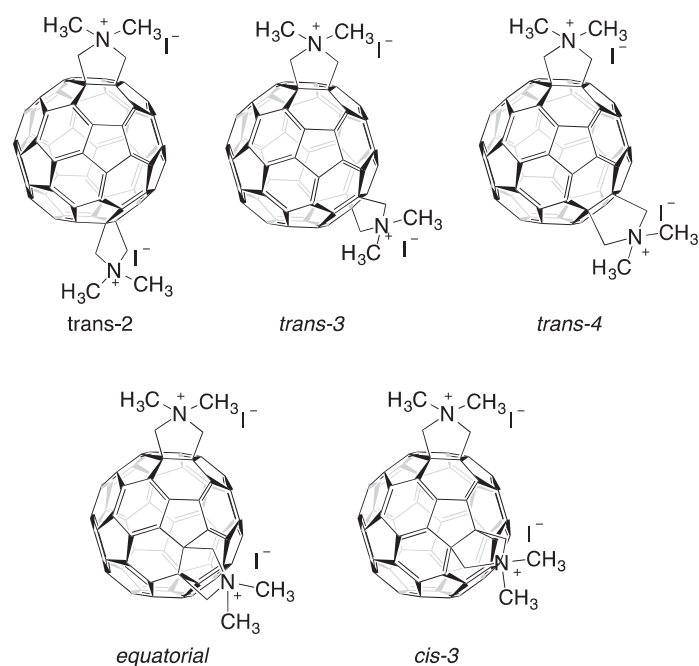
The conjugation of Hoechst 33258-hyaluronate fullerene (Ho-HF) was examined to evaluate its antitumor activity against HCT-116 cells via photodynamic treatment *in vitro*, exploiting Ho-HF conjugation to improve the PDT efficacy of fullerene [258]. Self-assembled microspheres containing fullerene-phenylalanine-poly lactic acid (C<sub>60</sub>-Phe-PLA) were synthesized in order to carry Mitroxantrone (MTX), an antitumor drug. The combination of sustained release of MTX and high photodynamic activity of C<sub>60</sub>-Phe-PLA demonstrated the potential of effective cancer treatment along with minimum side effects on normal tissues [259]. In a recent study, a chitosan oligosaccharide was grafted on fullerene in order to generate endogenous ROS in mitochondria in human malignant melanoma (A375) cells and results demonstrated that low dose of fullerene can encourage the generation of endogenous ROS [260].

The influence of fullerene nanoparticles on effectiveness of the microwave heating for cancer treatment has been reported, in this study the combination of microwave heating and C<sub>60</sub>

encapsulated in Pluronic F127-chitosan nanoparticles, a highly water soluble fullerene derivative which can be taken up by the cells and the improve the outcomes of the microwave hyperthermia for killing cancerous cells [261]. Various fullerene derivatives separately or in conjugation with other materials were utilized to assess their efficacy in photodynamic treatment of cancers: the overall results showed that fullerenes are promising nanoparticles be used in different cancer therapeutic approaches. Moreover, the combination with a number of antitumor drugs suggested that these constructs could be a promising approach in cancer therapy, even though further developments in this field requires more advanced investigations.

#### Anti HIV activity of fullerenes

HIV protease is the basic enzyme which assures virus survival and is specific for HIV proteins, so it is one of the main targets for antiviral treatments. The active site of HIV protease is a semi-opened hydrophobic ellipsoid, with Asp-25 and Asp-125 standing out on the surface of the cavity and catalyzing the protease function on the scissile peptide bond of the substrate. The diameter of cavity is 10 Å, close to the diameter of C<sub>60</sub> cage [39,262]. The antiviral activity of fullerene derivatives is mainly related to its molecular structure. Friedman *et al.* [262] reported that HIV protease can be inhibited introducing a fullerene molecule into the catalytic cavity [262,263]. Embedding C<sub>60</sub> sphere in the center of cavity allows Van der Waals interactions. The evaluation of antiviral activity of amphiphilic fullerene bisadducts prepared by Prato and coworkers showed different inhibition of HIV-1 [264]. They also reported that this inhibitory activity in fullerene derivatives is strongly related to functionalization sites. Marchesan *et al.* synthesized bis-fulleropyrrolidinium salts (Fig. 14) in order to evaluate their inhibitory activity against HIV-1 and HIV-2 [265],



**FIGURE 14**

Structures of fulleropyrrolidinium salts [265] to evaluate their inhibitory activity against HIV.

finding that the position of the side chains influences the antiviral activity, and *trans* fullerene bisadducts are more active than the *cis* one. Also Kotelnikova *et al.* reported that amino acid derivatives of fullerene are able to inhibit HIV [266].

Amino acid fullerene derivatives (ADFs) carrying bivalent metal ions penetrate through lipid bilayer of liposomes, insert to hydrophobic domains of proteins and therefore change the function of membrane bound enzymes. Cationic, anionic and amino acid derivatives of fullerene have been tested for antiviral activity by Mashino *et al.* and their results demonstrated that amino acid derivatives are the most active derivatives. The authors also compared HIV inhibition activity of fullerene derivatives with nevirapine (a clinical used HIV inhibitor) and they found that fullerene derivative is even more effective than nevirapine itself [267]. The anti-HIV activity against both HIV-1 and HIV-2 of fullerene derivatives of multiple carboxylic acid moieties was also evaluated by Troshin *et al.* [268]. They reported a fascinating approach using fullerene-sugar hybrid for photodegradation of HIV-1 protease. The role of the sugar was to promote the interactions between hybrid and HIV-1 protease via hydrogen bonding. UV/vis irradiation of hybrid-HIV-1P complex led to the virus degradation, which is attributed to generation of ROS by fullerene [268]. The enzymatic inhibition activity of TMA-C<sub>60</sub> was also investigated and proved by Yang *et al.* [269] in 2007; however, this inhibition probably did not have any relation to ROS production.

In summary, fullerene derivatives can complex and inhibit HIV protease based upon their structure, being able to bind in the cavity regions of HIV protease and inhibit virus replication significantly [270,271], and also on their ROS generation activity.

### Fullerene in drug delivery

Among the conventional methods for delivering drugs, oral and injection administration are the most used. In addition to these methods, there are some alternative approaches including pulmonary, transdermal, transmucosal, ocular administration and implantation [272]. By using drug delivery systems including nanoparticle carriers, the pharmacokinetics can be dramatically improved [273]. Transferring bioactive elements like proteins, DNAs, and also various small molecules through the membrane into cells (cellular delivery) drew considerable attention due to its essential role in medicine and drug delivery [274,275], and the possibility to reach the cell nucleus is even more limited by various barriers and really challenging [276]. Numerous nanoparticles can be used for targeted drug delivery and controlled release due to their biocompatibility and incredible characteristics, and fullerene exhibits a great potential in drug delivery systems [38,276,277].

Geometry, size and surface of fullerene cage seem to be appropriate for this application, considering its diameter in the range of 1 nm, approximately half of the average diameter of DNA helix [272]. Despite the hydrophobicity of fullerene cage, as already mentioned, it can be functionalized by hydrophilic moieties achieving water-solubility and used as drug carrier for bio-active molecules in biological environment. The first attempt in this field has been accomplished by Nakamura *et al.*, who synthesized two handed tetraminofullerenes which bound duplex DNA [278], therefore these DNA-fullerene nanoparticles are capable to enter

COS-1 cells. Foley *et al.* demonstrated that a fullerene derivative, C<sub>61</sub>(COOH)<sub>2</sub>, can cross the cell membrane and also localize into mitochondria, therefore it can be used in organelle-targeted drug delivery [133,277]. A fullerene derivative bearing two diamino side-chains was used for gene delivery to mammalian cells, entering the cell by endocytosis [279]. Rouse *et al.* proved that fullerene-based peptides are able to penetrate intact skin and, accordingly, can be applied in drug delivery [280], moreover the authors also found that mechanical flexion can increase the penetration rate of the nanoparticles into the dermis.

The main positive point in fullerene-based drug delivery systems is their ability to carry multiple drug payloads like taxol plus other chemotherapeutic drugs. This approach is really beneficial in cancer treatment, since cancer cells are easily drug-resistant and effective treatment often needs combination of more than one type of drug to overcome the resistance mechanism [281].

A drug delivery system containing fullerene and paclitaxel, ranging about 120–145 nm, was proposed by Zakharian *et al.* [282] in order to perform aerosol liposome delivery of paclitaxel for lung cancer treatment. Half-life of paclitaxel in bovine serum was 80 min and the paclitaxel release took place by enzymatic hydrolysis.

Buckysomes, the spherical nanostructure made of amphiphilic fullerenes which present many hydrophobic regions, can embed hydrophobic molecules like paclitaxel, a very hydrophobic anticancer drug, inside its hydrophobic pockets [283]. This spherical nanocarrier has been synthesized in aqueous environment and presents a diameter in order of 100–200 nm. Bucky-somes were compared to abraxane, which also have been used for delivering paclitaxel to suppress MCF-7 breast cancer cells. The results demonstrated that paclitaxel embedded bucky-somes can deliver higher amount of paclitaxel than abraxane [283,284].

A new class of water soluble fullerene-based transfecting agents has been synthesized using Hirsch–Bingel chemistry, and its ability in gene delivery has been evaluated: these derivatives can complex DNA, deliver it into cells and evoke gene expression [285].

Doxorubicin, as mentioned previously, is a precious anticancer drug but induces acute and chronic side effects. In order to mitigate its side effects one strategy is the use of drug delivery systems [10]. Fullerenes, due to their antioxidant and radical scavenging activity, have the potential to be conjugated with DOX and to mitigate the DOX side effects trigger by ROS. The challenge in this approach is that DOX is a water soluble while fullerene is hydrophobic, so Sun *et al.* used ethylene glycol spacers for conjugating methano-C<sub>60</sub> with DOX, improving the water solubility of the vector [286]. In a similar attempt fullerenols were conjugated to doxorubicin through a carbamate linker, in order to promote an efficient delivery [287]. This conjugation repressed the cancer cell proliferation *in vitro*, by blocking G2-M cell cycle, causing apoptosis. In addition, the fullereneol–doxorubicin conjugate exhibited high antitumor activity *in vivo* in a murine tumor model, without causing systemic toxicity like free DOX [287].

Recently, Prylutskyy *et al.* [257] have proven that fullerene acts as an interceptor for DOX and the hetero-complex of C<sub>60</sub>-DOX performs as a nano-carrier for delivering doxorubicin to target cells. The attractive point is that the conclusion derived from these

experiments can be generalized to other aromatic drugs like actinomycin D, mitoxantrone and topotecan [257].

An 'on-off' drug delivery system for cancer treatment has been developed by conjugation of DOX and fullerene ( $C_{60}$ ) following by attachment of the hydrophilic shell (distearoyl-sn-glycero-3-phosphoethanolamine-PEG-CNGRCK2HK3HK11, DSPE-PEG-NGR) to outer surface of the conjugation. This drug delivery system has strong stability in physiological solutions even with pH around 5.5 in the 'off' state, in contrast, in the 'on' state the generation of ROS by  $C_{60}$  results in the breaking of the ROS-sensitive linker which enables the exploding release of DOX. In this innovative drug delivery system, the combination of photodynamic therapy and chemotherapy could be applied against tumor cells [288].

Furthermore, fullerenes were conjugated to other species like nitroxide radicals to preserve normal tissue and cells against chemotherapy [37], they also can be conjugated to metals complexes, such as cisplatin, which is widely used for cancer chemotherapy [287,289]. Also in this case the anticancer activity against Lewis lung carcinoma cells of the conjugate was more efficient than the free drug [287].

In line with fullerene drug delivery applications, porphyrin adducts of cyclohexyl-fullerene were prepared for targeted delivery of  $Mg^{2+}$  to the heart muscles [290], which resulted in 80% recovery of tissue hypoxia symptoms less than 24 hours after a single injection. Release of  $Mg^{2+}$  by nanoparticles caused to stimulate ATP overproduction in oxygen depleted cells. Since these smart nanoparticles release  $Mg^{2+}$  cations just in reaction to the metabolic acidic shift, the positive changes in heart cell energy metabolism can help the treatment and preventing local myocardial hypoxic disorders and protect heart muscles in different hypoxia-caused clinical situations [290].

Fullerene also have been used in delivery of bioactive molecules like warfarin, a coumarin anticoagulant drug. Conjugation of warfarin to fullerene can alter its biological profile and prevent variation of its concentration in the blood [58,291]. Erythropoietin (EPO), a hormone mainly produced by kidneys, also can be linked to fullerenes and carbon nanotubes. EPO usually is administered through intravenous injection, although its biological activity is reduced abruptly in this route. Nanoparticulate system can be used for effective administration of EPO: for example EPO was absorbed on porous materials containing fullerene and its bioavailability was intensely enhanced compared to conventional administration [58,292].

The highly water-soluble and non-cytotoxic fullerene derivative, malonodiserinolamide  $C_{60}$  ( $C_{60}$ -Ser), was investigated as delivery system. The internalization of PF-633 fluorophore conjugated with  $C_{60}$ -Ser ( $C_{60}$ -SerPF) within living cancer cells was observed. In addition, *in vivo* experiments in mouse, bearing liver tumor, demonstrated that  $C_{60}$ -SerPF permeated through the vasculature of the tumor and it was detected in many tissues [293]. In another study, PEI-derivatized  $C_{60}$  was prepared by cationic polymerization of aziridine on the surface of  $C_{60}$ - $NH_2$ . Then, PEI- $C_{60}$  was decorated with folic acid (FA) via amide linker, and docetaxel (DTX) was conjugated to  $C_{60}$ -PEI-FA resulting in a complex drug delivery system [228], able to cross cell membranes and to cause apoptosis in tumor cells and exhibiting higher antitumor efficacy than free DXT, without toxic effects on normal tissues [294]. Recently, another fullerene-based drug

delivery system has been prepared via grafting hyaluronic acid (HA) onto fullerene and combining this with transferrin (Tf), which has antitumor efficacy and also can be used in photodynamic therapy [295]. Artesunate, an iron-dependent antimalarial drug, which is cytotoxic against tumor cells, was loaded into HA- $C_{60}$ -Tf with a high loading efficiency. The *in vitro* and *in vivo* evaluation of this system demonstrated its high antitumor efficacy [295].

#### Fullerenes in biosensors

A biosensor is an analytical device which can sense the presence and determine the amount of biomolecules, enzymes, microorganism, organelles, antibodies and receptors by combining a biological sensing element or a recognition site with a transducer [296,297]. The recognition site responds to the presence of the biomolecules and the transducer converts this to signals which are measurable [298].

Fullerenes can be used as mediator between recognition site and biosensor electrode, amplifying the electron transfer rate created by biochemical reactions of analyte and biological component in recognition site [298,299]. Considering that an efficient mediator should be hydrophilic and possess functional groups, which help it to conjugate the biomolecules in order to promote electron transfer at the reaction site, pristine fullerene does not seem to be the most advantageous mediator being hydrophobic and insoluble in polar media. However, fullerene derivatives can be effective material for using in biosensors [36,298]. Intensive research accomplished on  $C_{60}$  derivatives demonstrated that functionalization imparts characteristics which improve the efficacy in biosensor applications [299]. Among the other groups, carboxylic acid, amine and hydroxyl groups are beneficial in allowing the interactions between fullerenes with biomolecules [300]. The  $C_{60}$ -containing supported bilayer lipid membranes (s-BLMs) as molecular device have been investigated by Tien *et al.* [301] in 1997. The results showed that  $C_{60}$  inserted in BLM is an efficient electron mediator which can be used in biosensors. Following this investigation, Szymańska *et al.* used this method in an electrochemical sensor for the detection of neutral odorant and demonstrated that functionalized fullerene facilitates charge transmission [302]. Fullerene also have been used in glucose biosensing and overcome some limitations which exist in glucose determination [303]. The first attempt in this field was done in 2000 and various amounts of fullerene from 0.6 to 1.7  $\mu\text{m}$  were immobilized to amperometric biosensor against glucose oxidase enzyme. The results showed that, with the increasing of the fullerene amount, the sensitivity of biosensor also improved [304]. Later, a fullerene-cryptand-22 coated sensor was applied for sensing gluconic acid which is produced via glucose oxidation. The experiments demonstrated that this biosensor is selective to glucose and no interferences caused by biological samples were observed [305]. Furthermore, fullerene-cryptand-22 was used to coat piezoelectric quartz crystals along with immobilized  $C_{60}$ -urease membrane for sensing ammonium ions deriving from the catalytic hydrolysis of urea by urease [306]. Similar to this study, the  $C_{60}$ -coated piezoelectric quartz crystal sensor with immobilized  $C_{60}$ -lipase silicate plates was applied in order to detect optical isomer of amino acid esters. The synthesized sensor was capable to distinguish between L- and D-amino acid esters [307].

Zhilei *et al.*, with an inventive approach, developed a glucose biosensor exploiting fullerene along with ferrocene (Fc), chitosan (CS) and ionic liquid (IL) [308]. The efficacy of this biosensor was evaluated by chronoamperometry, cyclic voltammetry and impedance spectroscopy. The fast response of this biosensor and the minimum value of Michaelis–Menten Constant ( $k_m$ ) among other glucose biosensors demonstrated the high sensitivity and great performance of this biosensor. In this fullerene supports the oxidation of glucose molecules and stimulates the electrochemical reaction that results in the most applicable response in amperometric biosensors [308]. In 2012, novel Pd@Cys- $C_{60}$  nanoparticles were developed via *in situ* spontaneous reduction process, which was exploited for sensing glucose in glucose solution and human serum. The results showed that this biosensor has a linear range from 2.5  $\mu\text{M}$  to 1 mM and also no interferences from other biomolecules were reported [309]. A carboxyfullerene bisadduct,  $C_{60}[C(\text{COOH})_2]_2$ , possesses peroxide-like catalytic activity and can catalyze the reaction of peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of  $\text{H}_2\text{O}_2$ . Therefore,  $C_{60}[C(\text{COOH})_2]_2/\text{glucose oxidase (GOx)}/\text{TMB}$  system was designed to be a colorimetric sensor for glucose, which showed a detection range of 1.0–40  $\mu\text{M}$ . In addition, this highly sensitive colorimetric biosensor can be exploited for quantitative measurement of glucose in human serum [310]. In similar studies, functionalized fullerene was used as a mediator in glucose biosensors, exhibiting a convenient electron transfer between glucose oxide and glassy carbon [311,312]. Also Saeedfar *et al.* synthesized a urea biosensor using carboxyfullerene derivatives [313]. This biosensor contained fullerene–urea bioconjugate on an acrylic based hydrogen ion sensitive membrane, and demonstrated acceptable sensitiveness and response time.

In a recent study, a novel nanocomposite consisting of 3-amino-capto-1,2,4-triazole functionalized fullerene- $C_{60}$  coated by gold nanoparticles dispersed on a glassy nanostructured carbon electrode has been developed in order to investigate non-enzymatic sensing properties. This nanocomposite revealed excellent catalytic activity that is promising for application in glucose sensors with a really low detection limit [314]. However, considering the attractive properties of fullerenes, extensive investigations are needed to better exploit fullerene derivatives in the fabrication of biosensors, which remains an important field of application.

### Other biomedical applications

#### Fullerene for osteoporosis treatment

Osteoporosis is a disease which makes bones brittle and prone to fracture even with mild stresses. This happens when the bone density decreases and the bone tissue has become weaker and caused fracture in hip, spine and wrist commonly. It is well known that hydrophilic bisphosphonate groups have affinity towards the prominent bone mineral, hydroxyapatite, which is applicable in osteoporosis and other bone related diseases [39]. Fullerene was coupled to bisphosphonate to prepare a tissue-vectored compound, namely  $C_{60}(\text{OH})_{16}\text{AMBP}$ . The *in vitro* experiments demonstrated that this compound possesses good affinity for hydroxyapatite and reduces its mineralization by 50% at 1  $\mu\text{M}$  concentration [315]. Also  $C_{60}(\text{OH})_{30}$  reduces mineralization of hydroxyapatite (28% crystal growth rate) and exhibits affinity for hydroxyapatite [315]. Some studies reported that the reactive

oxygen species contribute in osteoclast differentiation begins with RANK-RANKL signals [316–318]. Therefore, controlling ROS can help in the treatment of osteoclast hyper-resorption on osteoarthritis. It has been proved that, in rat model, fullerene can reduce joint destruction and suppress bone resorption caused by osteoclasts [318]. Fullerenols also can stimulate osteogenesis in bone marrow, via eliminating ROS in models which experience oxidative stress caused by dexamethasone [319,320].

#### Antimicrobial activity of fullerenes

In 1990s, fullerenes have been evaluated for their antimicrobial activity and promising results on suppressive effects on bacteria including *Bacillus subtilis*, *Candida albicans* and *E. coli* were obtained [321,322]. The study on carboxyfullerene revealed that it can be exploited as an antimicrobial agent to suppress group A streptococcus infection [323]. The antimicrobial activity of carboxyfullerene was examined against *Streptococcus pyogenes* infection. *In vitro* experiments showed that this derivative can suppress growth of *S. pyogenes* and its administration to mice protects the 33% of them from death [323]. A fullerene peptide has been fabricated via solid-phase peptide synthesis from functionalized fullerene containing free amino group and *N*-Fmoc-L-glutamic acid  $\alpha$ -*tert*-butyl ester (Fig. 15) and its activity against bacteria, *Staphylococcus aureus* and *E. coli* was tested [324].

Tsao *et al.* in 2002 investigated the antimicrobial effect of carboxyfullerene on twenty bacteria strains [325]. The results revealed the role of this compound on inhibiting Gram-positive bacteria, while it had no effect on Gram-negative bacteria [325]. Photodynamic inactivation is an emerging antimicrobial approach that fullerene derivatives, especially water-soluble cationic ones, can facilitate upon irradiation, and in this case the compounds exhibit their activity both against Gram-positive and Gram negative bacteria [326].

In another study, a colloidal water-solution of  $C_{60}$  was prepared and the bacterial response to this  $nC_{60}$  preparation was examined [145]. Low concentration of  $nC_{60}$  inhibited the growth of Gram-positive and Gram-negative bacteria in various conditions [145]. Following this study, Lyon *et al.* investigated the antibacterial activity of  $nC_{60}$  and the influence of the aggregates morphology on the antibacterial activity using *B. subtilis* [327]. Therefore, they prepared fullerene water suspensions with four different strategies. The results displayed that the smaller fullerene aggregates, the higher antibacterial activity, this phenomenon could be related

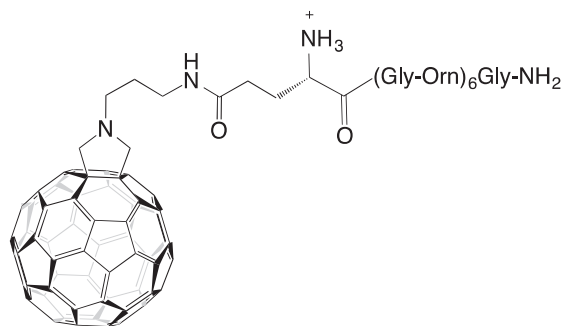


FIGURE 15

Schematic of functionalized fullerene containing free amino group and *N*-Fmoc-L-glutamic acid  $\alpha$ -*tert*-butyl ester.



to the surface area that in suspensions with smaller fullerene aggregates is greater and provides higher antibacterial activity [327].

The sub-lethal concentrations of colloidal aggregates of C<sub>60</sub> in water also can induce some adaptations in the Gram-negative *Pseudomonas putida* and Gram-positive *B. subtilis* bacteria including modification of membrane lipid composition, membrane fluidity and phase transition temperature [328].

The genotoxicity of fullerene on bacteria has been investigated in two distinct studies [206,329]. In the first fullerene did not demonstrate any genetic damage in *Salmonella typhimurium* and *E. coli* [206]. In contrast with this report, the latter study demonstrated DNA-damaging potential of fullerene towards *B. subtilis* by Rec-assay and umu test. Therefore, these results are not conclusive and DNA damage of fullerene was not caused by covalent DNA adduct formation, but there is an indirect mechanism which needs further investigations [329].

### Fullerenes as neuroprotective agent

Hyper-production of reactive oxygen and nitric oxide species, due to over excitation of glutamic acid receptors, can cause cell death resulting in chronic neurodegenerative diseases like Alzheimer's and Parkinson [39]. Neuroprotective activity of fullerene derivatives is similar to their radical scavenging activity. As mentioned above, fullerene and fullerenols have outstanding antioxidant activity. Their ability to reduce apoptosis in cortical neurons and also to block glutamic acid receptors have been reported [219,330]. Also the effectiveness of carboxyfullerenes in the treatment of amyotrophic lateral sclerosis (ALS) has been studied [331]. Hexasulfobutyl fullerenes and TMA fullerenes also demonstrated their ability to trap free radicals and play an efficient role in neurodegenerative diseases treatment [67,332]. Zha *et al.* investigated the correlation between fullerenes concentration and their activity [333]. At low concentrations, fullerenols exhibit neuroprotective effect and increase hippocampal neuronal viability, while at high concentrations, opposite results were observed and hippocampal neuronal viability was reduced, with consequent apoptosis.

The experimental allergic encephalomyelitis (EAE) [334] has been used as animal model to investigate the effect of fullerenes in neuroprotection [335]. It has been reported that a water-soluble fullerene derivative (ABS-75) bearing adamantyl units as NMDA receptor antagonist can block axonal damage and decrease progression in a chronic and progressive EAE model [335]. The role of fullerene in suppressing EAE suggests that the compound can be used as therapeutic strategy in controlling human multiple sclerosis [271]. Based upon various studies, water-soluble derivatives such as fullerenols and malonic acid fullerenes can react with hydroxyl and superoxide free radicals and reduce neuronal degeneration related to ROS [219].

### Fullerenes as MRI contrast agent for diagnosis

Magnetic resonance imaging is a technique, which uses gadolinium-based contrast agents. Fullerenes with their hollow cage can be used for encapsulating gadolinium in order to address challenges related to the use of Gd chelates for MRI [336], considering that after injection, C<sub>60</sub> distributes in tissues and relocates in selected organs without detectable and acute toxicity [39]. Endohedral

fullerenes, which entrap metal atoms in the molecular cage without releasing them even *in vivo*, are good candidates as contrast agent both in magnetic resonance imaging and X-ray imaging [38,39].

The first approach in using fullerene endohedrals in MRI was reported by Wilson *et al.* [337]. Gd@C<sub>60</sub>[C(COOH)<sub>2</sub>]<sub>10</sub> is water-soluble and it is easily uptaken in the reticuloendothelial system (RES). Later on Toth *et al.* also investigated the MRI applicability of Gd@C<sub>60</sub>(OH)<sub>x</sub> [338]. They showed that, being water proton relaxivities dependent on temperature, magnetic fields and also pH, these gadofullerenes possess the potential of pH-sensitive MRI contrast agent. Moreover Liu *et al.* investigated the potential of Gd<sup>3+</sup>-PEG-C<sub>60</sub> in both of photodynamic therapy and magnetic resonance imaging (MRI), the presence of Gd<sup>3+</sup> converts PEG-C<sub>60</sub> to a photosensitizer with dual application in diagnosis and treatment [246]. Shu *et al.* synthesized gadofullerene MRI contrast agent starting from higher fullerenes, as Gd@C<sub>82</sub>O<sub>6</sub>(OH)<sub>16</sub>(NHCH<sub>2</sub>CH<sub>2</sub>COOH)<sub>8</sub>, to evaluate its capability in magnetic resonance imaging and also in this case it was demonstrated that they have promising characteristics in this field [339–341].

### Fullerenes in cosmetics

Personal care and skin care products intricately intertwined with chemical industries. The emergence of nanotechnology in cosmetics triggered about 40 years ago with using liposomes in moisturizing creams [342]. Fullerenes, thanks to their antioxidant activity, are used in moisturizers [342]. For the first time, Inui *et al.* investigated the clinical efficacy of fullerenes for treating acne [343]. A fullerene-based gel was used in this study, decreasing the inflammatory lesions. Fullerene derivatives were reported to exhibit antioxidant activity and also anti-melanogenesis without any detectable cytotoxicity [344], and so they also have been used for anti-aging products. A water soluble fullerene complex consisting of PVP and C<sub>60</sub> has been proven for its cytoprotective activity against oxidative stress and its ultraviolet absorption proficiency. The PVP/C<sub>60</sub> is able to prevent the inhibitory effect of UVB on keratinocyte proliferation by its antioxidative effect that suggest the PVP/C<sub>60</sub> could be applied in cosmetic products due its free radical scavenging and as a barrier repair agent for promoting keratinocyte differentiation [345]. Despite these commercial uses, fullerenes may cause undesirable side effects, since application of fullerene in cosmetic products increase human exposure [346] and much more has to be investigated about this application.

An interesting role of fullerene nanomaterials which has been examined by the researchers is stimulation of hair growth. In this study a fullerene derivative has been developed and its role in treatment of hair loss has been examined. The results of experiments exhibited that in fullerene-treated mice the hair growth and also the number of hair follicles have increased compared to mice receiving only vehicle [347]. Production of reactive oxygen species as a possible underlying reason for aging and hair loss can describe the mechanism in which fullerene treats hair loss and increase the number of hair follicles, in fact, fullerene as a free radical scavenger is an attractive therapeutic option for treatment of diseases involving reactive oxygen species [347]. The various applications of fullerene derivatives in biomedicine are summarized in Table 1.

TABLE 1

**Fullerene derivatives and their various applications in biomedical engineering.**

Fullerene derivatives	Applications	Refs
Fullerenol or fullerol, C <sub>60</sub> (OH) <sub>12</sub>	Antioxidant and reducing apoptosis in cortical neurons cultures	[219]
Carboxyfullerene, C <sub>63</sub> ((COOH) <sub>2</sub> ) <sub>3</sub>	Inhibiting neurodegeneration involved in ALS	[331]
C <sub>60</sub> (ONO <sub>2</sub> ) <sub>7±2</sub>	With antioxidant activity can prevent oxidant-induced pulmonary diseases	[220]
Amino acid derivatives of fullerene	Antiviral activity	[267]
Hexa(sulfobutyl)fullerene, [C <sub>60</sub> (CH <sub>2</sub> -CH <sub>2</sub> -CH <sub>2</sub> -CH <sub>2</sub> SO <sub>3</sub> Na) <sub>6</sub> ](FC <sub>4</sub> S)]	Antiproliferative agent in the arteriosclerosis and inhibition of plasma lipid peroxidation	[222]
Fullerenol	Non-invasive image-guided cancer therapy	[348]
Fullerenol	Tumor inhibitory activity	[349]
bis-Fulleropyrrolidines	Inhibitory activity against HIV	[265]
Fullerenol	Anti-metastatic activity, cancer treatment	[287,350]
C <sub>60</sub> (Nd) nanoparticles	Inducing autophagy in cancer cells and enhancing chemotherapeutic killing of cancer cells	[351]
C <sub>60</sub> [>M(C <sub>3</sub> N <sub>6</sub> <sup>+</sup> C <sub>3</sub> ) <sub>2</sub> ][>M(C <sub>3</sub> N <sub>6</sub> C <sub>3</sub> ) <sub>2</sub> ](I <sup>-</sup> ) <sub>10</sub>	Antimicrobial photodynamic inactivation	[352]
tris-Malonic acid fullerenes	Radical scavenging	[332]
Dendrofullerene	Antioxidant activity	[224]
C <sub>60</sub> -PEI-FA	Drug delivery system	[294]
Fulleropyrrolidines bearing 3,5-di-tert-butyl-4-hydroxyphenyl	Antioxidant	[227]
Porphyrin-C <sub>60</sub>	PDT cell inactivation	[244]
C <sub>60</sub> -PEG-Gd	Anti-tumor activity	[246]
Pd@Cys-C <sub>60</sub> nanoparticles	Glucose biosensor	[309]
Cucurbit[8]uril-fullerene	Photodynamic therapy	[245]
Gd@C <sub>60</sub> [C(COOH) <sub>2</sub> ] <sub>10</sub>	MRI contrasts agent	[337]
6-Amino-g-cyclodextrin (ACD)/C <sub>60</sub>	Photodynamic therapy	[248]
Hexasulfobutyl fullerenes	Treatment of neurodegenerative diseases via radical scavenging	[332]
Fullerene-cryptand-22	Urea biosensor	[306]
Carboxyfullerene	Antimicrobial and antibacterial activity	[323,325]
C <sub>60</sub> (OH) <sub>16</sub> AMBP	Osteoporosis treatment	[315]
C <sub>60</sub> [C(COOH) <sub>2</sub> ] <sub>2</sub> /glucose oxidase (GOx)/TMB	Glucose colorimetric biosensor	[310]
Carboxyfullerene	Targeted drug delivery	[133,277]
Carboxyfullerene	Urea biosensor	[313]
Hoechst 33258-conjugated hyaluronated fullerene	Photodynamic tumor therapy	[258]
Fullerene-phenylalanine-poly lactic acid	Drug carrier – photodynamic therapy	[259]
Cystine C <sub>60</sub> derivative	Inhibiting apoptosis	[226]
Amphiliphilic fullerene bisadducts	Inhibiting HIV	[264]
DNA-fullerene nanoparticles	Carrying drug	[133,279]
Porphyrin adducts of cyclohexyl fullerene-C <sub>60</sub>	Targeted delivery of paramagnetic Mg <sup>2+</sup> isotope	[290]
Fullerenol	Stimulate osteogenesis in bone marrow	[319,320]
Fullerene containing free amino group and N-Fmoc-L-glutamic acid α-tert-butyl ester	Antibacterial activity	[324]
Malonodiserinamide derivatized fullerene	Drug delivery system	[293]
Carboxyfullerene	Antioxidant activity	[228]

**Conclusion and future outlook**

Fullerenes have become important molecules in different health-related subjects during the last three decades. The biomedical applications presented in this manuscript represent potential uses of this class of carbon family, due to its extraordinary properties

(see Table 1). As more applications of these nanomaterials become available, the demand for different forms of fullerenes will increase. For future works, functionalized fullerenes will considerably change the face of biological applications, and the credits go to the collaborative efforts of scientists who focus on both

chemistry and medicine. These collaborative efforts are critical for introducing the most effective modifications on the carbon cage for successful diagnosis and therapeutic outcomes in different biomedical applications. Here, we have provided an overview of the ever-expanding research on fullerenes in biomedical engineering applications ranging from drug delivery to noninvasive imaging in the human body. These studies are mostly limited to *in vitro* and *in vivo* researches and have not reached the final stages of clinical trials yet. It is expected that, with the rapid technological advancement in this field, this trend could change in the coming years so that the unique features of fullerenes could be exploited in clinical use. Although there are still major issues to overcome, the use of fullerenes for biomedical engineering will certainly have a bright future.

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